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Changes in Glial Cell Line Derived Neurotrophic Factor and Nerve Growth Factor Expression with Development, Age, Exercise and Hypertension

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CHANGES IN GLIAL CELL LINE DERIVED NEUROTROPHIC FACTOR AND
NERVE GROWTH FACTOR EXPRESSION WITH DEVELOPMENT,
AGE, EXERCISE AND HYPERTENSION

by

Bertha C. Rebimbas-Cohen

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CHANGES IN GLIAL CELL LINE DERIVED NEUROTROPHIC FACTOR AND
NERVE GROWTH FACTOR EXPRESSION WITH DEVELOPMENT,
AGE, EXERCISE AND HYPERTENSION

Bertha C. Rebimbas-Cohen, Ph.D.

Western Michigan University, 2005

Neurotrophic factors are important for neuronal growth, survival, and maintenance of cell phenotype. Glial cell line-derived neurotrophic factor (GDNF) affects sensory, autonomic and somatic motor neurons and nerve growth factor (NGF) affects sensory and sympathetic neurons. NGF and GDNF are produced by cardiac muscle and have potent effects on sensory and autonomic neuronal innervation of blood vessels. Neural innervation plays a key role in blood vessel function. Since neural innervation of blood vessels may be regulated by growth factors, we wanted to determine what changes would occur to NGF and GDNF levels with maturity, exercise and hypertension.

Changes in GDNF and NGF expression and innervation were examined in mesenteric vessels, hearts and in two models of hypertension, Dahl salt sensitive and Fischer 344 rats (L-NAME). Protein levels were examined using enzyme linked immunosorbant assay. Innervation density was studied using immunohistochemical methods. Increased pressure changes were examined using isolated cannulated vessel experiments.

GDNF levels were significantly higher in mesenteric veins versus mesenteric arteries. NGF levels were significantly higher in vessels of younger animals versus older animals. The localization of NGF and GDNF in blood vessels in sedentary animals was with fibers staining positive for tyrosine hydroxylase, indicating sympathetic innervation. In young or exercised animals NGF and GDNF were colocalized with calcitonin gene related peptide, indicating sensory innervation.

Blood pressure also decreased with exercise.

Exercise had little effect on the NGF protein content in atria or ventricle but led to an increase in GDNF content in the left and right atria and ventricles. Activity dependent regulation of trophic factor in the heart may play a role in altered nerve structure and function observed following exercise training.

Finally, GDNF expression changed with the development of hypertension. In both models of *in vivo* hypertension, GDNF protein content increased in the vessels of hypertensive animals. In *in vitro* studies, 4 hours of elevated pressure increased the GDNF protein content above controls.

These data suggest that both NGF and GDNF may be regulated in an exercise dependent manner, change with maturity, and may be a factor in the development or maintenance of hypertension.

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CHAPTER I

INTRODUCTION TO DISSERTATION

A variety of neurotrophic factors, including nerve growth factor (NGF) and glial cell line derived neurotrophic factor (GDNF) have been implicated in maintenance of neuronal cell bodies, proper innervation of the vascular wall (1), maintenance of neural plasticity (2-6), as well as influencing nerve growth in vivo (7-9). However, the mechanisms by which neurotrophic factors regulate the proper innervation to the vascular wall and cardiac tissue are not well understood. The studies described in this dissertation were aimed at elucidating the role that neurotrophic factors play in the development and maturation of sensory and motor innervation of cardiovascular tissues and whether alterations in neurotrophic factor expression or effects contribute to development of cardiovascular diseases like hypertension.

The development of the mammalian nervous system is dependent on the proper innervation of target tissues (e.g. tissues that neurons map to during development). Neurotrophic factors are important for neuronal survival maintenance of neuronal phenotype and maintenance of plasticity of neurons (2-6,10). The central neurotrophic factor hypothesis states that target organs secrete a substance that guides neurons to the target as well as ensuring the proper number of neurons survive and innervate the target (11).

In 1957, Levi-Montalcini discovered NGF and NGF has been shown to be necessary for survival and development of several types of neurons (12-30). NGF is now part of a family of proteins, termed neurotrophins, which play roles in the proper development of several types of neuronal populations (29,31-37). In mammals there are four neurotrophins, NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT 4). All four neurotrophins appear to be derived from a common ancestral gene similar in sequence and structure (38).

In addition to the neurotrophins there is a second group of growth factors termed neurotrophic factors that play a role in nervous system development and in the maintenance of the adult nervous system(39-48). Neurotrophic factors come from a variety of sources. They can be synthesized and released by organs targeted by

sensory and sympathetic neurons, neighboring neurons, as well as from the sensory and sympathetic neurons themselves. As a result, neurotrophic factors can have their actions described as target derived trophic factors or paracrine or autocrine factors.

NGF signals in neuronal tissue via a slow dissociation tyrosine kinase receptor (trk-A), triggering tyrosine phosphorylation (1,49,50). This occurs on the tyrosine residues within the intracellular portion of the receptor. Tyrosine phosphorylation is essential to the signaling mechanisms of this factor which will ultimately play a role in allowing neurons to survive, grow, and differentiate. Once phosphorylated, trk-A then mediates ras activation and sets in motion a survival pathway involving phosphatidylinositol-3-kinase activity (51). This in turn will create phosphoinositide intermediates which activate the serine/threonine kinase, Akt/PKB (52). This causes the activation of tyrosine kinase, phospholipase C γ (PLC γ) and SHC(53). Activation of the serine-threonine kinase pathway is essential for cell survival and proliferation (52,54). NGF and its receptor is then transported in a retrograde manner to the nerve cell body, where it exerts its effects by initiating signaling via either the ras, cdc-42/ras/rho, G protein families, MAP kinase, PI-3 kinase, and Jun kinase cascades or numerous other signaling cascades (1,23,55). In addition to the trk receptor, NGF also signals through a low affinity receptor called p75 NTR. This receptor is considered low affinity due to its rapid association and dissociation with NGF. Signaling through p75 in the absence of trk-A, usually causes the initiation of an apoptotic signal in several types of cells both neuronal and nonneuronal (56-59). In the presence of trkA receptor, p75 causes enhanced neurotrophin responsiveness, leading to a signaling pathway that promotes survival or differentiation.

During early development, several neuronal populations are dependent on NGF; i.e. cerebellar granule cells, dorsal root ganglia (sensory neurons), superior cervical ganglion (sympathetic neurons), cortical and hippocampal neurons, oligodendrites and schwann cells (53). Following the developmental stages, in which mapping and neurite pruning occur, cells may continue their dependence on NGF for the life of the organism (60), or may become responsive to other trophic factors, such

as BDNF or GDNF. Although NGF has mainly been deemed important during development, NGF's importance persists throughout life and affects glia, nonneuronal cells, as well as serving in neuroprotective and repair functions(6,61,62). In adulthood, approximately one-half of nociceptive nerves are dependent on NGF for trophic support, whereas the other half are sensitive to GDNF(43,63,64) thus suggesting that as nerves mature or develop there is the possibility that nervous system dependence in the form of fiber type switching may occur.

Similar to NGF, the neurotrophic factor GDNF exerts many lasting and important effects on both the developing and adult mammalian nervous system. GDNF was originally identified as a survival factor for midbrain dopaminergic neurons in 1993 by Lin et al. (65). GDNF is important in the development of many types of peripheral neurons (45,60,66,67). GDNF and its family of neurotrophic factors, which include neurturin (NRTN), artemin (ARTN) and persephin (PSPN), maintain several neuronal populations in the central and peripheral nervous systems (68-75) and are structurally similar to transforming growth factor β . GDNF, NRTN, and ARTN were all seen to support the survival of both peripheral and central neurons, whereas PSPN was seen to only support the survival of central and motor neurons (46,76). GDNF is produced by neuronal and nonneuronal cells and binds to GDNF receptor alpha (GFR α) (72,77-81), while other GDNF family members bind to GFR α 2(NRTN), GFR α 3(ARTN), GFR α 4(PSPN). GFR α forms a homodimeric complex, which then signals through the RET receptor tyrosine kinase. The RET receptor tyrosine kinase activates SHC adaptor proteins which will activate ras, ultimately causing cell proliferation and survival. In all cells except motoneurons, GDNF requires transforming growth factor beta for initiating the transfer of GFR α 1 to the cell membrane(48). GDNF is also able to signal following complex formation with GFR α 1, via heparan sulphate glycosaminoglycan, which activates the Met receptor tyrosine kinase through cytoplasmic Src-family kinases. GDNF may also signal through the neural cell adhesion molecule (NCAM) in cells lacking RET(82-84). GDNF first binds to NCAM and GFR α 1 to form a complex. This complex will

then activate the tyrosine kinases Fyn and FAK. This causes Schwann cell migration and axonal growth via GDNF signaling independent of RET(83).

In the peripheral nervous system, GDNF affects sensory, autonomic and somatic motor neurons(69,85-87). GDNF also plays a role in the development of dorsal root sensory neurons, primarily thought to be responsive to NGF in the developmental stages of neural growth (63). GDNF has been implicated in regulating presynaptic branching and continuous synaptic remodeling(88), and is involved in control of parasympathetic innervation and in the development of dorsal root sensory neurons (63,88).

Both NGF and GDNF play an important role in innervating cardiovascular tissues. In the heart, NGF derived from cardiac myocytes regulates sympathetic innervation (89). The amount of NGF produced directly correlates with the innervation density of the target tissue. On the other hand, hyperinnervation has been linked to sudden cardiac death(90). Therefore, ample amounts of neurotrophic factor are necessary for proper innervation of the cardiac tissue during development, but can be a detriment if overexpressed. In addition to cardiac tissue (91), innervation of coronary arteries also requires the presence of NGF(92).

Similar to NGF, GDNF and neurturin are required for sympathetic axons to enter target tissues as well as mediating parasympathetic innervation into target tissues (45,93). In blood vessels, artemin, a member of the GDNF family, has been shown to direct sympathetic neurons along blood vessels to their targets (44). Taken together, these data suggest that neurotrophic factors are critical in development and maintenance of the nervous system and therefore changes in the expression or content may have profound effects on the overall physiological functions of the organism. Deciphering the role neurotrophic factors play in the innervation of cardiovascular tissues is an important step to knowing how these molecules will affect function of the system in general once the neurotrophic factors become altered, such as in hypertension.

Hypertension afflicts nearly 50 million Americans (American Heart Association: 2004 Heart and Stroke Statistical Update). Hypertension has been characterized by an increase in sympathetic innervation and hyperplasia of vascular smooth muscle cells (94-96). Increased sympathetic innervation may cause increased constriction of the vasculature, which leads to increased blood pressure(95-97). Increases in NGF levels have been linked to hyperinnervation of the vasculature (37) and data obtained from studies done in the spontaneously hypertensive rat (SHR) model suggest that increases in sympathetic innervation of vascular tissues in this model may be a result of alterations in expression of NGF(96,98-101). This hypothesis is supported by studies demonstrating that normotensive rats exposed to increased levels of exogenous NGF develop hyperinnervation of the vascular wall, increased number and size of sympathetic neurons innervating the vessel wall, as well as hypertrophy of the smooth muscle cells(94,102). Studies conducted using neonatal SHRs suggest that administration of anti-NGF, can prevent hyperinnervation of vascular tissues and development of hypertension in this animal model(103).

Similar events to what occurs in hypertension occur in bladder innervation patterns during bladder obstruction. In bladder obstruction, in which the end result is that the bladder and smooth muscle cells stretch, there is an increase in smooth muscle cell growth and changes in innervation patterns, which have been closely linked to changes in NGF content (104). These changes closely mimic changes observed in vascular tissues of SHRs with the development of hypertension. This suggests that as the trophic factor content increases the density of innervation increases which may ultimately lead to constriction of the vasculature and an increase in overall blood pressure.

NGF has been linked to hypertension, but the role GDNF could play in hypertension has yet not been explored. GDNF has been found to be involved in inducing hypertensive-like behavior of cells in other diseases. One example is infantile hypertrophic pyloric stenosis (IHPS). In IHPS, the smooth muscle cells of the pylori become hypertrophic with progression of the disease and begin to increase

their production of GDNF(105-107). The increased production of GDNF has been linked to progression of the disease. This disease closely mimics the actions of hypertension. This suggests that, like in hypertension, increases in GDNF may play a role in the development or maintenance of the disease. In addition, the increase in GDNF may allow for increases in sympathetic innervation which may translate into increased vasoconstriction of the vasculature and an ultimate increase in blood pressure. In IHPS, like in the SHR, GDNF may also be acting as a cue to increase innervation to the vascular via the vascular smooth muscle cells. As hypertrophy is a hallmark of hypertension, overexpression of GDNF may help to cause and maintain hyperinnervation of the vasculature a similar pattern to that seen in NGF in the vasculature of the SHR.

Another organ in which expression of NGF and GDNF is important is in the heart. NGF and GDNF are produced by cardiac muscle(108-113) and both have potent effects on autonomic and sensory neurons. Altered expression of neurotrophic factors can have effects on nerve integrity, innervation pattern, and nervous system function in cardiac neurons. In the heart sympathetic innervation is maintained in part by NGF and parasympathetic innervation is maintained in part by GDNF(86,90,108,114). GDNF and NGF acting on cardiac tissue have been shown to alter neurotransmitter release and density of target innervation, leading to both long and short term effects on the heart (1,8,46,66,115-119). Thus changes in neurotrophic factor content may play a critical role in how the heart reinnervates allografts or after denervation.

Neurotrophic factors have been the subject of much intense research over the last 50 years. Changes in neurotrophic factor production and release by target tissue can have profound effects on the neural tissues and thus can have huge effects on overall organismal physiology. Of these neurotrophic factors, this dissertation will concentrate on the roles of NGF and GDNF in development, maturation of innervation of cardiovascular tissues and changes with disease, i.e. hypertension. I propose that levels of expression of the neurotrophic factors NGF and GDNF in

cardiovascular tissues will be very dynamic during development and in disease states and that changes in neurotrophic factor expression will lead to alterations in the structure and function of neurons innervating cardiovascular tissues.

The studies in this dissertation chose to address the following aims: To determine whether the trophic factors GDNF and NGF were present in the cardiovascular tissues, i.e. mesenteric vasculature and cardiac tissue. 2. To determine which part of the nervous system was this neurotrophic factor associated with. 3. To determine if innervation patterns change with development and exercise. 4. To determine if GDNF protein expression changes with the development of hypertension.

To address these specific aims the following studies were performed. For determining GDNF and NGF protein content in developing animals, animals were allowed to mature and sacrificed at two time points to determine changes in protein content over time and changes in neural density. In the same study, a second group of animals were subjected to voluntarily exercise (a running wheel) and the protein content and neural density was examined for these animals as well. Finally, two separate sets of experiments were performed using two models of hypertension to determine GDNF protein content, density of fibers and possible mechanism of action in the mesenteric vasculature.

My data indicate that as rats mature there is a decrease in the amount of protein content quantified in the mesenteric vasculature. In addition, the incidence of sedentary behavior may be linked to increases in sympathetic fiber density which has been closely linked to increases in blood pressure. My data also suggest that via a moderate exercise regimen these less desirable effects, increased sympathetic innervation and increased blood pressure, were able to be reversed to a more favorable phenotype associated with more sensory innervation, implicating decreased blood pressure and overall better physical wellbeing. Finally, my in vitro data suggest that as little as four hours of elevated pressure may be enough to cause an

increase in trophic factor content and these changes may be responsible for alterations seen in the development hypertension.

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INTRODUCTION TO CHAPTER II

In the peripheral nervous system, Glial cell line-derived neurotrophic factor (GDNF) affects sensory, autonomic and somatic motor neurons. GDNF also plays a role in the development of dorsal root sensory neurons, primarily thought to be responsive to NGF in the developmental stages of neural growth (1). GDNF has been implicated in regulating presynaptic branching (2) and continuous synaptic remodeling, and is involved in control of parasympathetic innervation and in the development of dorsal root sensory neurons (1,2).

The sympathetic nervous system is maintained in part by the neurotrophin, NGF (3-5). NGF has also been linked to maintenance of sensory neurons (1,6) and increases in NGF expression have been linked to increases in sympathetic and sensory innervation of target tissue (7,8). Thus NGF is an important regulator of both sympathetic, as well as, sensory nervous system development, maintenance, innervation, and mapping of nerve fibers to target tissue.

In the heart the sympathetic nervous system is maintained by NGF and the parasympathetic nervous system is maintained by GDNF. The structure and function of neurons innervating a given target tissue may be dependent on trophic factor expression by that tissue.

Recent studies have shown that there are different populations of sympathetic neurons innervating mesenteric arteries and veins. However the role that neurotrophic factors play in innervation of mesenteric vessels is not well understood. In our studies we chose to address several factors: We wanted to address the following questions: Are GDNF and NGF present in the vasculature? If so, do their expressions change with age, exercise or hypertension? and finally, 3. Does expression of GDNF and NGF change in the heart with age, or exercise?

Several studies have shown that there is an age related decline in trophic factor. It has been well established that NGF expression in dorsal root ganglion, middle cerebral arteries, basal forebrain neurons, hippocampal neurons, spinal motoneurons and aorta decreases with age(9-14). Nerve fiber loss in cerebral blood vessels (10) and decreased nerve regeneration have been shown with increased age

(15). Several studies have shown levels of expression of NGF mRNA (16) and protein (17) to decline in cardiac muscle to low stable levels of expression by 3 weeks of age, at which time the degree of sympathetic innervation also reaches adult levels (18). However there are no studies establishing a decrease in GDNF expression with age although there is precedence with other trophic factors for this to occur. Our studies chose to address the question, does GDNF and NGF change expression with age in the mesenteric bed? In addition, if there were changes over time would a change in behavior, i.e. exercise or sedentary behavior, have any effect on trophic factor expression?

Exercise training has been shown to have beneficial effects on sympathetic nervous system function and overall cardiovascular health (19-21). Several studies have also implicated that trophic factor expression, namely BDNF, was upregulated with exercise causing a defense mechanism against cell death and motor function degeneration, and an upregulation of brain repair functions(22,23). We have shown exercise training to increase neurotrophic factor expression in skeletal muscle (24) and cardiac muscle (25,26). In the healthy heart, exercise training leads to an increase in cardiac output, athletic hypertrophy of the left ventricle, resting bradycardia. The current study was undertaken to determine whether affects neurotrophic factor expression in vascular tissue as well as cardiac tissue. If exercise alters vascular neurotrophic factor expression, then this could be responsible for changes in the sympathetic nervous system function observed with exercise. Our study chose to address whether increased physical activity would affect GDNF and NGF expression in the vasculature and cardiac tissue and provide the same beneficial affects as seen with other trophic factors. If GDNF 's role in the heart is to support the parasympathetic nervous system (5,6), then elevated expression of GDNF with exercise may enhance parasympathetic nervous system function, which may help to lower blood pressure. Both NGF (42) and GDNF (36) have been shown to play a role in reinnervation of denervated cardiac muscle. If levels of expression of NGF and GDNF are increased with exercise, then it is possible that an exercise-training

program could facilitate sympathetic and parasympathetic reinnervation of cardiac tissues.

Finally we chose to address whether changes in trophic factor expression could be associated with diseased states, namely hypertension. Hypertension is characterized in spontaneously hypertensive rats (SHR) by hypertrophy, hyperplasia and hyperinnervation of vascular smooth muscle cells in the resistance vessel walls (1,2,27,28). Increases in NGF expression, in the vasculature, have been associated with hypertension (17,29). During development of hypertension, vascular smooth muscle cells undergo hyperplasia causing them to become hyperresponsive to neural stimulation (28). In spontaneously hypertensive rats (SHR) increased levels of NGF expression have been linked to hyperinnervation of the vasculature (6,27,30). GDNF also acts on receptors in autonomic neuronal populations and sympathetic ganglia, supporting fiber growth in peripheral ganglia (31,32). Studies conducted after dorsal root injury indicated that with chronic administration of GDNF, sensory axons were able to restore connections with their target tissue, restoring sensory function (2).

Infantile hypertrophic pyloric stenosis (IHPS) is a narrowing of the pylorus of the stomach caused by smooth muscle thickening. Studies conducted in IHPS indicate that smooth muscle hypertrophy any hyperplasia are associated with increased production of GDNF (33). As hypertrophy is a hallmark of hypertension, we propose that the smooth muscle cells lining the walls of the vasculature will increase their GDNF expression as they become hypertrophic. Overexpression of GDNF may alter structure and function in several neuronal populations that innervate the vasculature. Taken together these data suggest that GDNF can stimulate the growth of axons, both sympathetic and sensory, to target tissue supporting fiber outgrowth, which may lead to increased vasoconstriction causing a feedback loop that stimulates a further increase in blood pressure.

In order to understand blood pressure regulation, the mechanisms regulating innervation to the smooth muscle in the vasculature is paramount. Understanding the effects of increased or decreased expression of neurotrophic factors by vascular

smooth muscle and changes in innervation in the vascular beds helps to increase our knowledge of the basic neural regulation of blood vessel tone. Insight into neurotrophic factor expression in the vasculature is important because neuronal control of resistance vessels exemplifies one of the major controlling factors for regulation of blood pressure. In addition to gaining knowledge into some of the basic differences between how arteries and veins regulate blood flow, results of these studies may provide some answers into the mechanism of hypertension development, and ways to ameliorate it via noninvasive solutions such as exercise.

In the first chapter we will begin by addressing the following basic questions: 1. Is GDNF found in the mesenteric bed? 2. Which part of the nervous system does it appear to be utilized by and does fiber density change with age and exercise? 3. Will exercise alter GDNF protein expression?

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CHAPTER II

GLIAL CELL LINE DERIVED NEUROTROPHIC FACTOR MAY PLAY A ROLE IN NERVOUS SYSTEM DEVELOPMENT OF THE MESENTERIC ARTERIES AND VEINS

Abstract:

In the peripheral nervous system, Glial cell line-derived neurotrophic factor (GDNF) affects sensory, autonomic and somatic motor neurons. The structure and function of neurons innervating a given target tissue may be dependent on trophic factor expression by that tissue. Recent studies have shown that there are different populations of sympathetic neurons innervating mesenteric arteries and veins. However the role that neurotrophic factors play in innervation of mesenteric vessels is not well understood. We hypothesize that GDNF levels in the mesenteric arteries will be lower than in mesenteric veins and this may underlie the different patterns of innervation in these target tissues. The differences observed may be responsible for the different innervation patterns seen in this vascular bed. The objective of this study was to determine whether GDNF is differentially expressed in the mesenteric arteries and veins of Fisher 344 rats.

Following one week of acclimation eight animals were euthanized and mesenteric arteries and veins were removed and analyzed for GDNF content by enzyme-linked immunosorbant assay. Patterns of innervation and distribution of GDNF in mesenteric arteries and veins were examined using immunohistochemical methods. Results indicated that GDNF expression is associated with vascular smooth muscle in the mesenteric artery and vein. In mesenteric veins, GDNF positive nerve fibers were infrequently observed. In contrast, the mesenteric artery displayed frequent fibers staining positive for GDNF. GDNF levels as evaluated by ELISA were significantly higher in mesenteric veins versus mesenteric arteries in 8 wk old versus 22 wk old animals. The results indicate that GDNF protein content is higher in the mesenteric veins of 8 wk versus 22 wk old animals. Smooth muscles cells of both arteries and veins stain positively for GDNF. GDNF protein content decreased 10 fold between 8 and 22 wks of age denoting a possible role for GDNF initially in development. There was an increase in sympathetic (Tyrosine Hydroxylase) innervation between 8 and 22 wks of age. GDNF was found to be colocalized to sympathetic fibers in 22 wk old animals, but not in 8 wk old animals. The shift in neural density from a balanced sensory-sympathetic innervation to an increase in

sympathetic and decrease in sensory innervation may account for the different patterns of innervation seen between the arteries and veins. The greater shift in increased sympathetic innervation in the veins versus arteries may indicate a role for the veins in regulating blood pressure as we age and/or become sedentary.

Introduction:

Neurotrophic factors are important for neuronal growth, neuronal survival, maintenance of nerve cell phenotype, and maintenance of neural plasticity (1-5). A variety of neurotrophic factors, including nerve growth factor (NGF) and GDNF, have been implicated in the maintenance of neuronal cell bodies and proper innervation of target tissue (6).

The sympathetic nervous system is maintained in part by the neurotrophin, NGF (6-8). Increases in NGF expression have been linked to increases in sympathetic and sensory innervation (9,10). Increased levels of expression of NGF have also been linked to hyperinnervation of the vasculature (11).

GDNF is important in the growth and preservation of central and peripheral neurons, is most prominently expressed in the gut, and is essential for the development of the enteric nervous system (12,13). GDNF signals through the Ret/Gfr α 1 receptor complex in autonomic neuronal populations and sympathetic ganglia, supporting fiber growth in peripheral ganglia (14). GDNF also plays a role in the development of dorsal root sensory neurons, primarily thought to be responsive to NGF in the developmental stages of neural growth (15). GDNF has been implicated in regulating presynaptic branching (16) and continuous synaptic remodeling, and is involved in control of parasympathetic innervation and in the development of dorsal root sensory neurons (15,16). Studies conducted after dorsal root injury indicated that with chronic administration of GDNF, sensory axons were able to restore connections with their target tissue, restoring sensory function (16). Thus, growth factors are important in stimulating the growth of axons, both sympathetic and sensory, to target tissue supporting fiber outgrowth, which may alter overall vascular function.

There is evidence that blood vessels give off cues to axons to migrate towards blood vessels (17). Initial studies done in low-density dissociated cultures of sympathetic neurons of mouse cervical and thoracic sympathetic ganglia showed that a member of the GDNF family, Artemin, promoted the growth and proliferation of sympathetic neuroblasts (18). Recently Honma et. al (19), showed that Artemin was found to be a vascular derived neurotrophic factor for sympathetic neurons. In addition, during migration and axonal mapping, Artemin which was found in smooth muscle cells of blood vessels, acted as a guidance factor to encourage growth of sympathetic axonal projections toward the blood vessels (19).

GDNF is required for formation of the enteric nervous system (12,13), but there is controversy as to whether GDNF plays a role in innervation of the vasculature of the mesentery and if so does its expression change with age and exercise. Thus, we chose to determine whether patterns of vascular innervation change with age in young adult rats and whether increased physical activity can slow or reverse changes seen with age. Results of preliminary studies from our laboratory suggested that vascular smooth muscle cells in culture secrete GDNF (20). Thus the aim of these studies was to determine if GDNF was found in vascular tissue and to determine if GDNF was available to act as a target derived neurotrophic factor for neurons innervating vascular tissues.

Materials and Methods:

Subjects:

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. Twenty four Fisher 344 rats (Charles River, Portage MI) were used. Eight animals were sacrificed at 8 weeks of age (8 wk Sed), 8 were maintained for an additional 14 weeks without access to running wheels (22 wk Sed) and 8 were maintained for an additional 14 weeks with access to running wheels (22 wk VT). Animals were housed singly in Nalgene® cages with access to food and water *ad*

libitum. Animals were kept on a 12:12 hour light-dark cycle in a room where the temperature was regulated (22-24°C). All animals were monitored daily and body weights were measured weekly to ensure positive weight gain.

General Tissue Collection and Processing for GDNF:

Animals were sacrificed via CO₂ asphyxiation followed by thoracotomy. Mesenteric arteries and veins were removed and cleaned of any fat and connective tissue. Vessels were either flash frozen by contact with dry ice and stored at -80°C for later determination of GDNF content or placed in Zamboni's fixative and kept at 4°C prior to immunohistochemical processing.

Tissue Processing for Determination of GDNF Content:

Just prior to processing, frozen vessel samples were dipped into liquid nitrogen and then crushed on a metal block chilled on dry ice. The pulverized vessel 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 while being homogenized for 30 seconds using a variable speed Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). Homogenate was centrifuged at 13,000xg and the supernatant was analyzed using an enzyme-linked immunosorbant assay (ELISA) specific for GDNF.

Enzyme-Linked Immunosorbant Assay (ELISA) for GDNF:

Ninety six well plates (NUNC-ImmunoTM, Rochester, NY) were coated with 100 µl/well of 1 µg/ml anti-GDNF monoclonal antibody (R & D Systems, Minneapolis, MN) in PBS, pH 7.4, overnight at room temperature (RT) in a humidified chamber. Plates were washed 2 X 1 minute with 250 µl/well of wash buffer containing 0.4 M NaCl and 0.05% Tween-20 in 0.1 M PBS, pH 7.4.

Remaining sites on the plate were blocked with 200 μ l/well of 1.0% BSA, 5% sucrose in PBS for 1 hour at RT in a humidified chamber. Plates were washed 3 times. Following the third wash, 100 μ l/well of GDNF standard or sample was added. Fresh standards were made for each assay and were diluted in sample buffer. Each sample and standard was assayed in quadruplicate. Plates containing sample and GDNF standard were incubated for 2 hours at RT in a humidified chamber. The plates were washed. Each well then received 100 μ l of biotinylated anti-GDNF 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 were washed 3 times. Each well then received 100 μ l of streptavidin linked β -galactosidase (100 μ g/ml, Molecular Probes, Eugene, OR) diluted in TBS containing 1% BSA and 0.05% Tween-20 and were placed in a humidified chamber and incubated at RT for 20 minutes. The plates were washed. During the final wash, the galactosidase substrate chlorophenolred-beta-D-galactopyranoside (CPRG, Roche, Palo Alto CA) was dissolved in substrate buffer consisting of 0.1 M sodium phosphate and 1 mM $MgCl_2$ at pH 7.4 with 1% BSA. Plates were incubated in substrate buffer with CPRG overnight, at room temperature, in a humidified chamber. Absorbance was measured at 575, using a microplate spectrophotometer.

Immunohistochemistry in Whole Mount Blood Vessels:

After the mesenteric arteries and veins were removed and cleaned of any fat or connective tissue, the vessels were fixed overnight in Zamboni's fixative solution (2% Paraformaldehyde, 0.15% Picric Acid, in 0.1 M Phosphate Buffer) at 25°C. The tissue was washed 3 times for 10 minutes in dimethylsulfoxide. The tissue was then washed 3 times for 10 minutes in PBS. For GDNF experiments, the tissue was blocked for an hour at 37°C in 10% mouse serum diluted with PBS containing 1% BSA. The tissue was incubated overnight at 25°C in primary antibody (Rabbit anti-GDNF; 1:200 dilution, Santa Cruz, Santa Cruz, CA) in PBS-BSA 1%. The tissue

was then washed 3 times for 5 minutes in PBS. Tissue was incubated for an hour at 37°C in secondary antibody (Mouse anti-Rabbit IgG biotin conjugate; 1:1500 dilution, Sigma, St. Louis, MO) in PBS-BSA 1 %. The tissue was washed 3 times for 5 minutes in PBS. The tissue was incubated 30 minutes in PBS-BSA 1% containing fluorophore (streptavidin conjugated to Alexa Fluor 488 or 594; 1:1000 dilution, Molecular Probes, Eugene, OR). The tissue was washed 3 times for 5 minutes in PBS. The tissue was coverslipped, stored in 50% PBS-Glycerol at 4°C and was visualized on a confocal microscope. Controls for immunohistochemical analysis consisted of omission of anti-GDNF primary antibody and also the use of non-immune rabbit serum as the primary antibody to identify any non-specific staining. Vessels were also stained for the following markers: calcitonin gene related peptide ((CGRP) Rabbit anti-CGRP 1:8000 dilution in PBS-BSA 1%, Sigma, St. Louis, MO) and Tyrosine Hydroxylase ((TH)Rabbit anti-TH 1:500 dilution in PBS-BSA 1%, Chemicon, Temecula, CA and smooth muscle cell specific α -actin ((SMCA)Mouse anti-SMCA 1:250 dilution in PBS-BSA 1%, Sigma, St. Louis, MO). Following an overnight wash, the second marker was applied and staining proceeded as previously stated with the following exceptions: α -actin did not require a secondary antibody because it was directly conjugated to Fluorescein isothiocyanate (FITC). The tissue was coverslipped and visualized on a confocal microscope. Filter settings for Alexa Fluor 488 and FITC were band pass 505-530 and Alexa Fluor 594 were band pass 585-615. Samples were optically sectioned in 2 μ m slices, 20 slices/image, and then optically reconstructed via LSM® software to attain a projection encompassing all layers of the vessel.

Blood Pressure Measurements:

Systolic blood pressure was measured using tail-cuff plethysmography (IITC. Inc., Woodland Hills, CA). Blood pressure information from the amplifier was recorded on a chart recorder (Gould Instrument Systems Inc., Dayton, OH). Animals were restrained in tubes in a heated chamber (29 ° C) and given 30 minutes to acclimate prior to blood pressure measurement.

Neurite Density Analysis:

Changes in neurite density were determined by placing a grid over each blood vessel image, as captured by the LSM software, and then counting the number of times neurites crossed the grid (21).

Statistical Analysis:

Comparisons between 8 wk Sed, 22 wk old sedentary and 22 wk VT animals were done using an analysis of variance followed by a least significant difference (LSD) post hoc analysis. For all tests significance was set to $p < 0.05$. All data values are reported as the mean \pm standard error of the mean.

Results:

Gross Observations:

Body weights of 8 wk Sed animals (170.3 ± 2.9 g) were significantly different from the 22 wk Sed (274.8 ± 3.8 g) and 22 wk VT animals (260.4 ± 4.5 g). All animals showed positive weight gain throughout the study. Following tissue removal, it was noted that the mesenteric vessels from 8 wk Sedentary and 22 wk VT animals had a very healthy and lean appearance in contrast to the 22 wk Sed animals, which had large deposits of fat and connective tissue surrounding the vessels.

Distances Run:

For voluntary running, 8 week old rats were introduced to cages with running wheels. The average distance run by the rats steadily increased over a 5 week time period, stabilizing at 250 ± 75 m/day (1.7 ± 0.5 km/week = 1.1 ± 0.3 miles per week).

Blood Pressure:

Eight week old sedentary rats had a systolic blood pressure of 129.6 ± 3.2 mm Hg. By 22 weeks of age systolic blood pressure in sedentary rats was significantly

elevated (146.7 ± 1.9 mm Hg) compared to that in 8 week old sedentary rats. Fourteen weeks of voluntary exercise caused a significant decrease in systolic blood pressure (124.14 ± 3.2 mm Hg) compared to that in age matched 22 wk sedentary rats, $p < 0.05$.

GDNF Protein Content in Mesenteric Arteries and Veins:

GDNF protein content in mesenteric veins (2131.3 ± 89.8 pg GDNF/vessel; $n = 8$) of 8 week old animals was significantly higher than that in 8 week old mesenteric arteries (1639.5 ± 80.6 pg GDNF/vessel; $n = 6$, $p < 0.05$ (Figure 1). In 22 week old sedentary animals, GDNF protein content in the mesenteric veins (170.8 ± 19.5 pg GDNF/vessel; $n = 6$) was significantly higher than that in mesenteric arteries (77.7 ± 1.9 pg GDNF/vessel; $n = 5$), $p < 0.05$ (Figure 1). Between 8 and 22 weeks of age there was a ten fold decrease in GDNF protein content in arteries and veins. Following 14 weeks of exercise, GDNF protein content in the mesenteric arteries was significantly higher (123.2 ± 9.1 pg GDNF/vessel; $n = 5$) than that in mesenteric veins (86.4 ± 5.7 pg GDNF/vessel; $n = 5$), $p < 0.05$ (Figure 2). In addition, GDNF protein content was also significantly higher in the mesenteric artery of 22 week old voluntary animals versus 22 week old sedentary animals. In the mesenteric vein we found the opposite to be true. Following exercise, the GDNF protein content in 22 week old voluntary animals was significantly lower than that of 22 week old sedentary animals, $p < 0.05$.

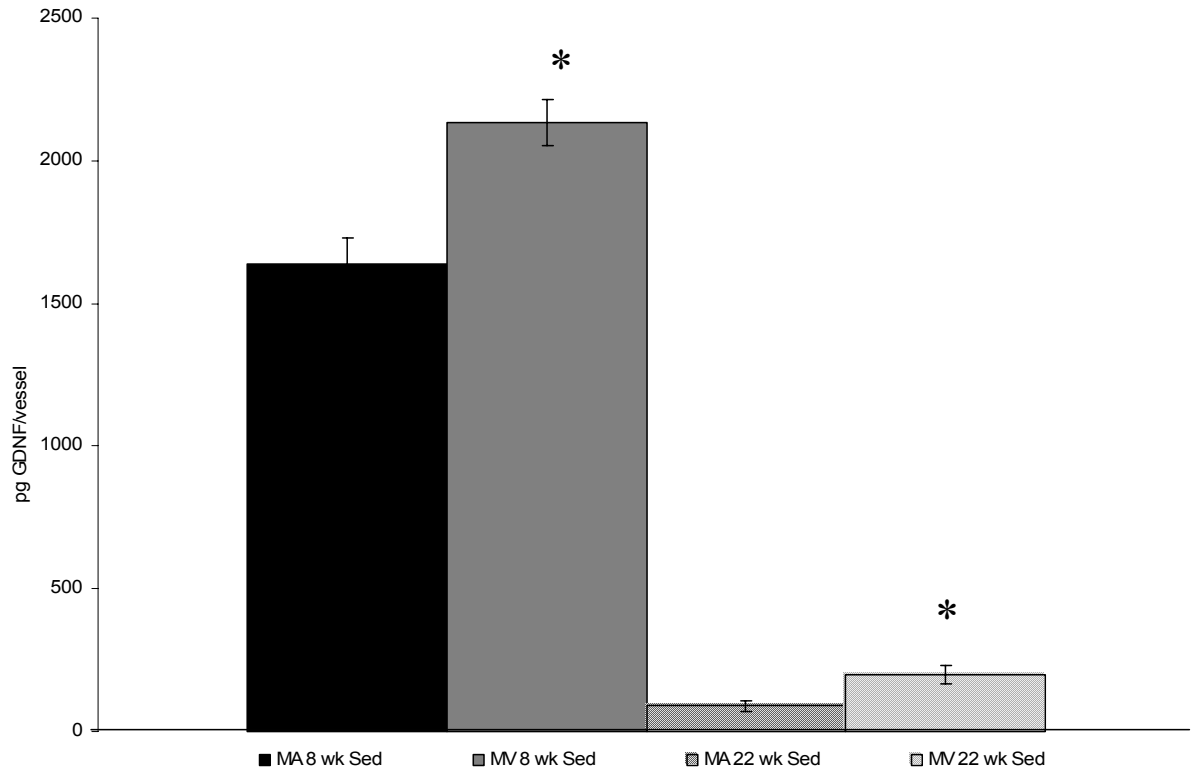


Figure 1: GDNF Protein Content in 8 Wk Versus 22 Wk Old Sedentary Animals. GDNF protein content in 8 (n=7-8) and 22 (n = 5-6) week old sedentary animals in the mesenteric veins is significantly higher than that in mesenteric arteries (*) ($p < 0.05$, ANOVA, SNK). The GDNF protein content was decreased in both artery and vein in the older animals by a ten-fold decrease.

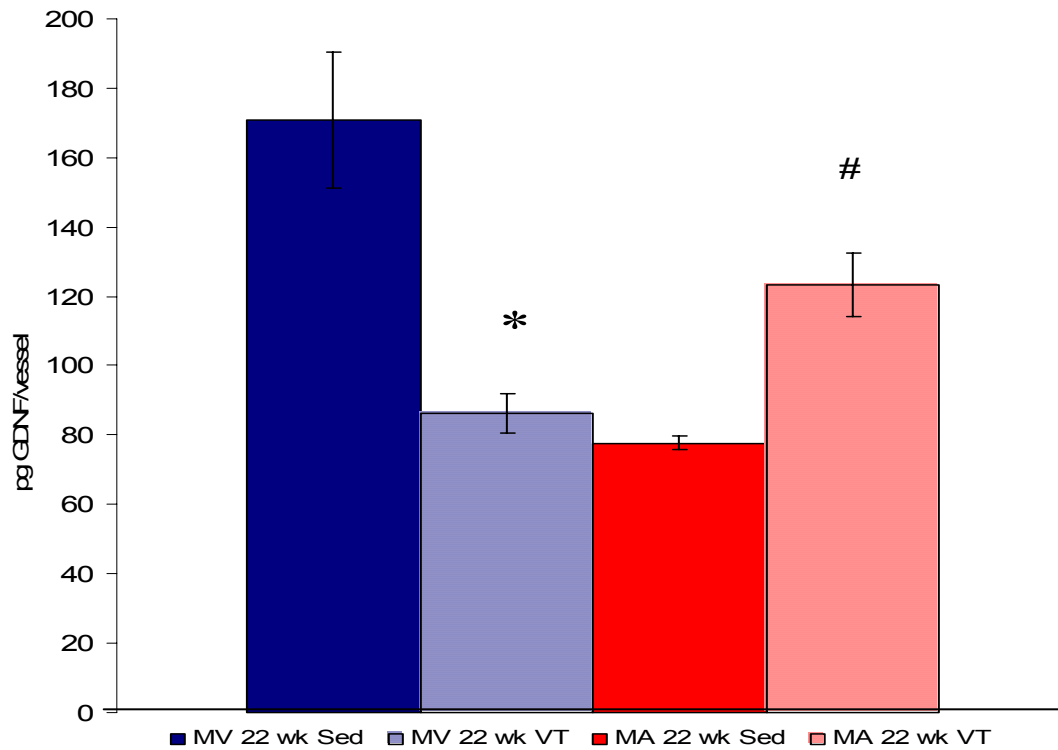


Figure 2: GDNF Protein Content in 22 Wk Old Sedentary Animals Versus 22 Wk Old Exercised Animals. GDNF protein content in 22 wk old sedentary (Sed; n = 6)(*) vein is significantly higher than that in 22 wk old exercised (VT; n = 8) week old animals. GDNF protein content in 22 wk old sedentary (Sed; n = 5)(#) artery is significantly less than that in 22wk old exercised (VT; n = 5) week old animals. (p<0.05, ANOVA, LSD).

GDNF Localization in Vessels of 8 and 22 Wk Old Animals:

Immunohistochemical studies were done to test which type of nerves contained GDNF. Sympathetic nerve fibers were identified using antibodies against TH and sensory nerve fibers were identified using antibodies against CGRP.

Vessels from 8 Week Old Sedentary Animals:

The arteries and veins from 8 week old animals showed fibers staining positive for GDNF, CGRP and TH. Fibers staining positive for GDNF were infrequently seen in the veins (n=8). GDNF was also observed to be colocalized to vascular smooth muscle cells in both arteries and veins (n=8) (Figure 3A). In these vessels, GDNF and TH were not colocalized and appeared as independent fibers from one another (Figure 3B), whereas the fibers staining positive for CGRP and GDNF appeared as one fiber (Figure 3C). In both arteries and veins, fibers staining positive for TH and CGRP were in close proximity to one another, but not contained within the same fiber (Figure 3D).

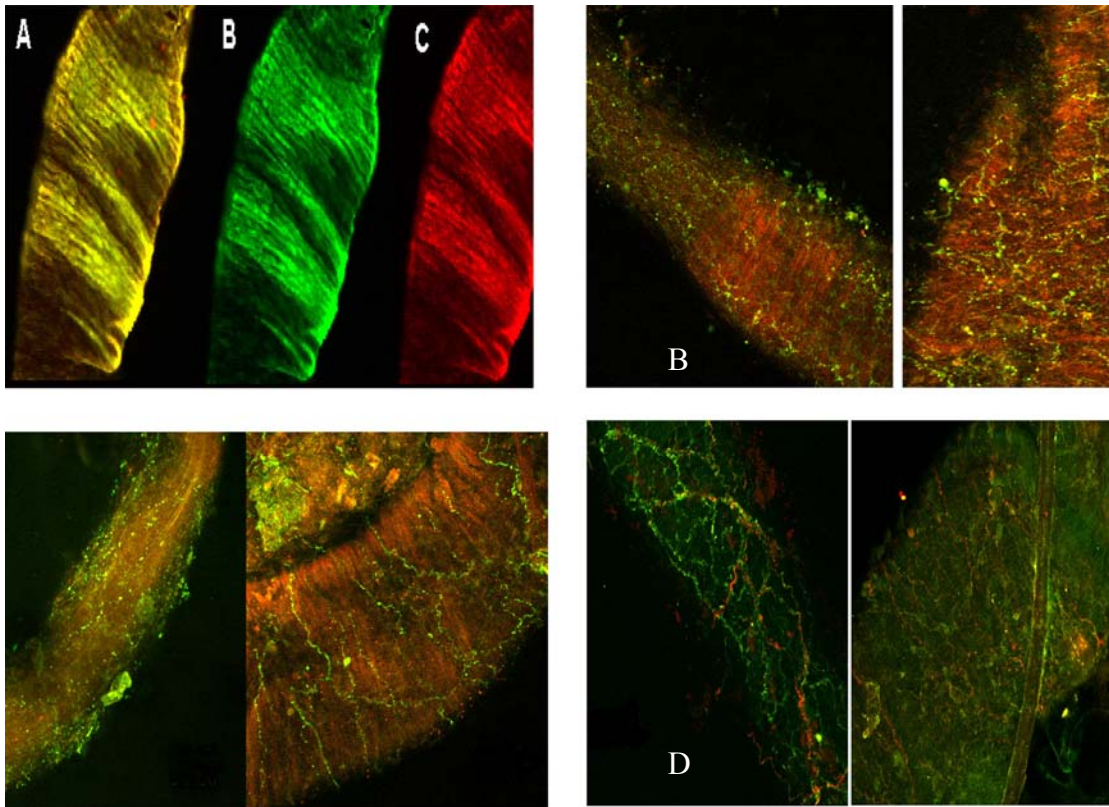


Figure 3: Immunohistochemical Staining of Vessels from 8 Wk Old Sedentary Animals. A.) A mesenteric vein stained for GDNF (red), α -actin (green) and the overlay (yellow). B.) A mesenteric artery (right) and mesenteric vein (left) stained with antibodies against GDNF (red) and TH (green). C.) A mesenteric artery (right) and mesenteric vein (left) stained with antibodies against GDNF (red) and CGRP (green). D.) A mesenteric artery (right) and mesenteric vein (left) stained with antibodies against CGRP (red) and TH (green).

Vessels from 22 Week Old Sedentary Animals:

Staining in the arteries of 22 wk old sedentary animals showed fibers staining positive for GDNF, CGRP and TH. In contrast to the 8 wk old sedentary animals, fibers staining positive for TH and GDNF were colocalized within the same fibers (Figure 4B), whereas the fibers staining positive for GDNF and CGRP appeared as two separate fibers (4C). In both arteries and veins, fibers staining positive for TH and CGRP were in close proximity to one another, but not contained within the same fiber (Figure 4D).

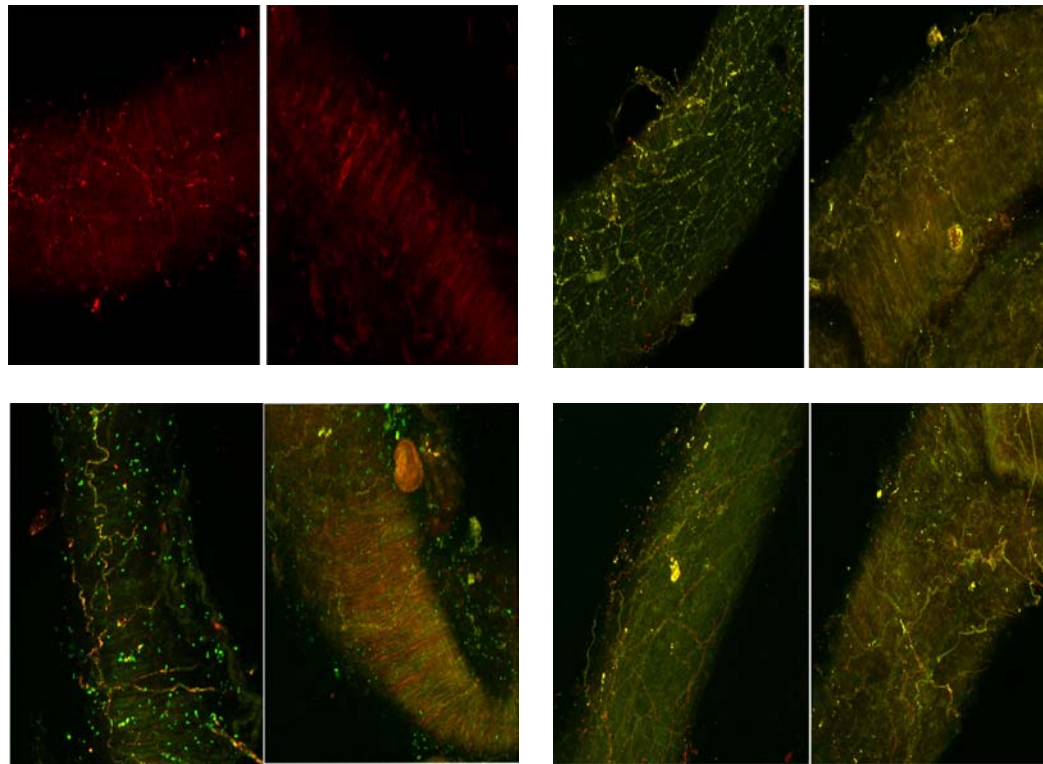


Figure 4: Immunohistochemical Staining of Vessels from 22 Wk Old Sedentary Animals. A.) A mesenteric artery (right) and vein (left) stained for GDNF (red). B.) A mesenteric artery (right) and mesenteric vein (left) stained with antibodies against GDNF (red) and TH (green). C) A mesenteric artery (right) and mesenteric vein (left) stained with antibodies against GDNF (red) and CGRP (green). D.) A mesenteric artery (right) and mesenteric vein (left) stained with antibodies against CGRP (red) and TH (green).

Vessels from 22 Week Old Exercised Animals:

Staining in the arteries of 22 week old exercised animals showed positive fiber staining for GDNF, CGRP and TH. In these vessels, GDNF and TH were once again not colocalized and appeared as independent fibers from one another, similar to that seen in the 8 week old sedentary animals (Figure 5B), whereas the fibers staining positive for CGRP and GDNF appeared as one fiber (Figure 5A). In both arteries and veins, fibers staining positive for TH and CGRP were in close proximity to one another, but not contained within the same fiber (data not shown).

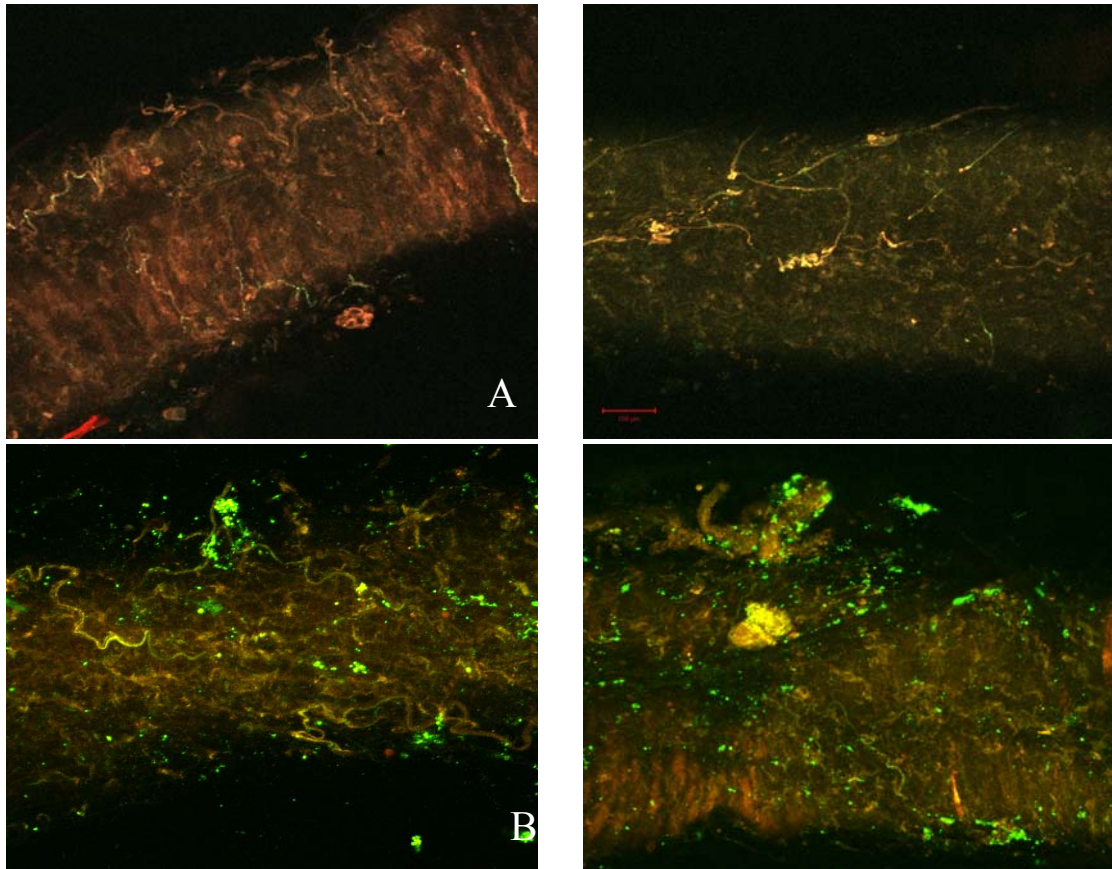


Figure 5: Immunohistochemical Staining of Vessels from 22 Wk Old Exercised Animals. Right panels are mesenteric arteries and left panels are mesenteric veins A.) Vessels were stained with antibodies against GDNF (red) and CGRP (green). B.) Vessels were stained with antibodies against GDNF (red) and TH (green). The panels indicate that the vessels of older exercised animals show a shift in colocalization of GDNF and CGRP, a marker for sensory innervation, to one of GDNF and TH, a marker for sympathetic innervation.

Nerve Fiber Density:

Veins of 8 Week Old Sedentary Animals Versus 22 Week Old Sedentary Animals:

In the veins of 22 week old sedentary animals (36 grid crossings/194 $\mu\text{m}^2 \pm 3$), the incidence of fibers staining positive for GDNF was decreased compared to that in 8 week old sedentary animals (73.6 grid crossings/194 $\mu\text{m}^2 \pm 18.7$) (Figure 6B). The number of fibers staining positive for TH increased in 22 week old sedentary animals (137.25 grid crossings/194 μm^2) versus 8 week old sedentary animals (93.75 grid crossings/194 μm^2) (Figure 6B). The fibers staining positive for CGRP in 22 week old sedentary animals (52.5 grid crossings/194 μm^2) decreased from 8 week old sedentary animals 72.5 grid crossings/194 μm^2 .

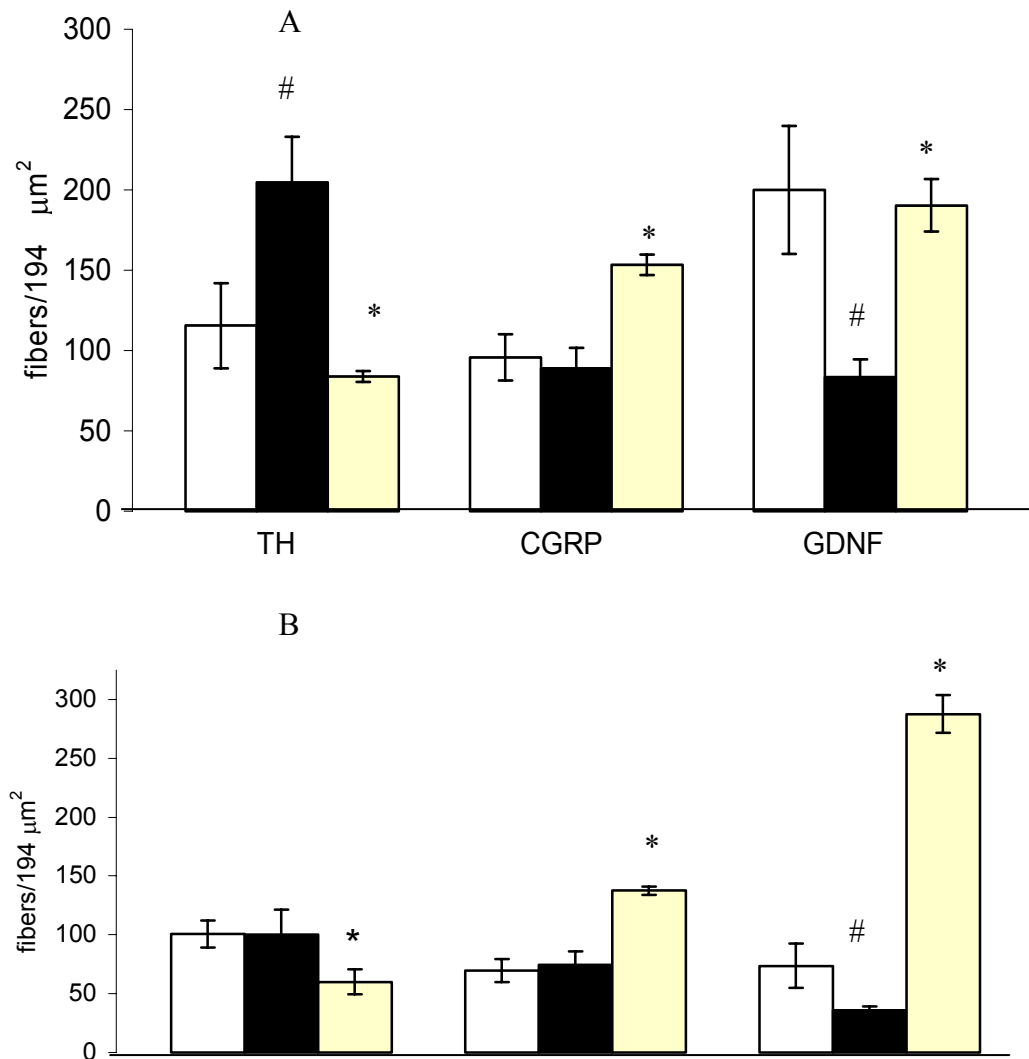


Figure 6: Summary of Data Assessing Sensory and Sympathetic Innervation in Mesenteric Vessels. All fiber crossings are grid crossings/194 μm^2 . In mesenteric arteries (A), the density of TH positive fibers significantly increased between 8 and 22 (#) wk sedentary animals. Over the same period of time GDNF positive fibers significantly decreased and there was no change in the density of CGRP positive fibers. Exercise caused a significant decrease in TH positive fibers (*), as well as a significant increase in CGRP and GDNF positive fibers (*) compared to 22 wk old sedentary animals. In the mesenteric veins (B), while the density of TH positive fibers and CGRP positive fibers did not change between 8 and 22 wks of age, GDNF positive fiber density significantly decreased between 8 and 22 (#) week sedentary animals. Exercise caused a significant decrease in TH positive fibers (*) as well as a significant increase in CGRP (*) and GDNF positive fibers (*) compared to 22 wk old sedentary animals. Significance is defined as $p < 0.05$, ANOVA, LSD.

Veins of 22 Week Old Sedentary Animals Versus 22 Week Old Exercised

Animals:

Following 14 weeks of exercise, there was a significant decrease in TH positive fibers (60 grid crossings/194 $\mu\text{m}^2 \pm 10.5$) as well as a significant increase in CGRP (137.5 grid crossings/194 $\mu\text{m}^2 \pm 3.7$) and GDNF positive fibers (287.6 grid crossings/194 $\mu\text{m}^2 \pm 16$) compared to vessels from age matched sedentary controls (Figure 6B).

Arteries of 8 Week Old Sedentary Animals Versus 22 Week Old Sedentary

Animals:

In mesenteric arteries, TH positive fiber density significantly increased between 8 (115.5 grid crossings/194 $\mu\text{m}^2 \pm 26.5$) and 22 weeks of age (204.7 grid crossings/194 $\mu\text{m}^2 \pm 28$) in sedentary animals (Figure 6A). GDNF positive fiber density significantly decreased between 8 (200 grid crossings/194 $\mu\text{m}^2 \pm 39.8$) and 22 weeks of age (83.3 grid crossings/194 $\mu\text{m}^2 \pm 11.2$) in sedentary animals.

Arteries of 22 Week Old Sedentary Animals Versus 22 Week Old Exercised

Animals:

Following 14 weeks of exercise, there was a significant decrease in TH positive fibers from 204.7 grid crossings/194 $\mu\text{m}^2 \pm 28$ to 84 grid crossings/194 $\mu\text{m}^2 \pm 3.3$ in vessels from age-matched sedentary controls. There was also a significant increase in CGRP positive fibers from 89 grid crossings/194 $\mu\text{m}^2 \pm 12.8$ to 153.2 ± 6.4 grid crossings/194 μm^2 in vessels from age-matched sedentary controls. GDNF positive fibers increased from 83.3 grid crossings/194 $\mu\text{m}^2 \pm 11.2$ to 190 grid crossings/194 $\mu\text{m}^2 \pm 16.1$ from age matched controls (Figure 6A).

Discussion:

In the current study, we have demonstrated new findings concerning GDNF expression and innervation in the mesenteric vascular bed. The GDNF protein content in the mesenteric vessels decreased between 8 and 22 weeks of age in

sedentary rats. Voluntary exercise resulted in a lowering of blood pressure and increased GDNF protein expression in mesenteric arteries. In addition, we have demonstrated that GDNF protein content is higher in the veins of 8 and 22 wk old sedentary animals compared to that in mesenteric arteries. In mesenteric vessels from 22 week sedentary old rats, GDNF was found to colocalize with TH, a marker of sympathetic innervation. GDNF was found to be colocalized with CGRP, a marker for sensory innervation in vessels from 8 week old sedentary and 22 week old exercised rats. Taken together, these data suggest that as we age, innervation patterns may shift from a balanced sensory/sympathetic innervation pattern seen in the younger healthier animals to a predominantly sympathetic innervation pattern in older animals. The findings that GDNF protein content of mesenteric vessels undergoes, a 10 fold decrease between 8 and 22 weeks of age, may be indicative of GDNF's function in the developmental stages of innervation in these vessels.

In the current study we have demonstrated that GDNF protein expression was high in a young animal, decreased with age and sedentary behavior and was once again increased with exercise in mesenteric arteries. Although there have been no studies in the vasculature indicating that GDNF decreases with aging, there is precedence for other trophic factors decreasing with age. It has been well established that NGF expression in dorsal root ganglion, middle cerebral arteries, basal forebrain neurons, hippocampal neurons, spinal motoneurons and aorta decreases with age (22-27). Nerve fiber loss in cerebral blood vessels (23) and decreased nerve regeneration have been shown with increased age (28). In the present study we have shown that GDNF protein content in mesenteric arteries and veins decrease between 8 and 22 weeks of age. We also found that blood pressure increased with age over the same period of time. Following exercise GDNF content of mesenteric arteries was increased, while blood pressure was decreased back to levels similar to those seen in 8 week old animals. This decrease in GDNF expression may have implications for survival of neurons and neural plasticity. This decrease may be responsible for the decline in neurotrophic factor in peripheral ganglia and may help account for neuronal atrophy that is observed in the elderly.

When neural density was assessed in the current study there were differences in innervation patterns seen between the artery and vein. In studies conducted by Browning et al.(29), it was observed that there are different populations of neurons that innervate the arteries and veins. There also have been several studies in which arteries and veins responded differently to a variety of stimuli (30-32). In the current study we have observed that GDNF was found to be colocalized to the smooth muscle cells of the vascular walls in both the arteries and veins. When fiber density was assessed we observed that the 22 week old sedentary arteries contained twice as many TH positive fibers as compared to the veins. It is possible that the differing levels of neurotrophic factor expression found in artery and vein may underlie the different patterns of innervation and neural function in these tissues.

Although neurons require neurotrophic factors for development, as the neurons age the dependency on neurotrophic factors may change. In a study conducted with IB4-binding neurons, neurons switched from dependence on NGF in embryonic life to dependence on GDNF in postnatal life (33) and in the vestibular ganglion, neurons switch from NT3 to GDNF (34). Our data showing the pattern of change of GDNF colocalization with CGRP positive fibers at 8 weeks, colocalization with TH in a 22 week old sedentary animal and then again with CGRP at 22 weeks of age following exercise, suggests that an underlying possibility that the sensory fibers may have sustained dependence on GDNF to maintain the sensory innervation mediated through signaling by a target-derived trophic factor such as GDNF in this vascular bed. In contrast, the colocalization of trophic factor and a sympathetic marker may signify a possible shift in the sensitivity of nerves to GDNF from sensory to sympathetic nerves.

Results of our study illustrate the potential beneficial effects of exercise on the nervous system. Exercise has been shown to upregulate trophic content in rat hippocampal neurons in aged animals, back to levels which parallel those seen in younger rats (35). We have demonstrated that GDNF protein content is higher in the vessels of 22 week old exercised animals compared to vessels from 22 week old sedentary animals. We also showed that the density of fibers staining positive for both GDNF and CGRP is significantly greater in the exercised animals versus the sedentary

animals, while blood pressures were significantly lower in exercised animals compared to the sedentary age-matched controls.

These findings may have great implications for helping to ameliorate conditions seen with aging and disease and highlight changes that lifestyle choices may play in dictating vascular wellness. Instead of medication or surgery, beneficial effects to the nervous system may be implemented by modifications in lifestyle. More work is required to decipher the role GDNF may play in neural remodeling and regulation of vascular innervation.

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INTRODUCTION TO CHAPTER III

Following the analysis of GDNF in the vasculature, we wanted to investigate if the changes in trophic factor expression and innervation patterns were similar with nerve growth factor (NGF). Neurotrophic factors are important for neuronal growth, survival, and maintenance of cell phenotype. NGF affects sensory and sympathetic neurons. The aim of the current study was to determine whether levels of expression of NGF differ in arteries and veins that receive dense innervation and whether NGF levels change with aging and with exercise. Following one week of acclimation eight animals were euthanized and mesenteric arteries and veins were removed and analyzed for NGF content via ELISA. Following 14 weeks of following voluntary running in a running wheel eight animals were euthanized, along with eight age-matched sedentary controls and NGF protein levels were analyzed. NGF protein levels were significantly higher in arteries and veins of 8-wk-old animals versus arteries and veins of 22-wk-old sedentary animals. NGF protein content in arteries and veins from 22-wk-VT animals was significantly greater than that in 22-wk-Sed animals. The localization and density of NGF-positive fibers in blood vessels was examined using immunohistochemical methods. NGF-positive fiber crossings/194 μm^2 in arteries and veins in 8-week-old animals were significantly higher than arteries and veins of 22-week-old animals. Following 14 weeks of voluntary exercise the NGF protein content significantly increased between 22 week sedentary versus 22 week voluntary animals in both artery and vein. The results show that NGF protein levels are higher in the arteries and veins of younger animals versus older animals. In addition, when older animals were exercised the NGF protein content increased. This may imply that protein expression may be mediated in an exercise dependent manner. This change in trophic factor content and expression in the vessels at different points of development may underlie the different patterns of innervation seen in these target tissues and possible changes in function, such as changes in blood pressure regulation, observed in the vascular bed as we age or exercise.

CHAPTER III

VOLUNTARY EXERCISE ALTERS NERVE GROWTH FACTOR EXPRESSION AND INNERVATION IN MESENTERIC ARTERIES AND VEINS

Abstract:

Neurotrophic factors are important for neuronal growth, survival, and maintenance of cell phenotype. Nerve Growth Factor (NGF) affects sensory and sympathetic neurons. The aim of the current study was to determine whether levels of expression of NGF differ in arteries and veins that receive dense innervation and whether NGF levels change with aging and with exercise. Following one week of acclimation, eight animals were euthanized and mesenteric arteries and veins were removed and analyzed for NGF content by enzyme-linked immunosorbant assay. NGF protein levels were significantly higher in arteries and veins of 8-wk-old animals versus arteries and veins of 22-wk-old sedentary animals. NGF protein content in arteries and veins from 22-wk-VT animals was significantly greater than that in 22-wk-Sed animals. The localization and density of NGF-positive fibers in blood vessels was examined using immunohistochemical methods. NGF-positive fiber crossings/194 μm^2 in arteries and veins in 8-week-old animals were significantly higher than arteries and veins of 22-week-old animals. The results show that NGF protein levels are higher in the arteries and veins of younger animals versus older animals. In addition, when mature animals are exercised, the NGF protein content increases. This change in trophic factor content and expression in the vessels at different points of development may underlie the different patterns of innervation seen in these target tissues and possible changes in function, such as changes in blood pressure regulation, observed in the vascular bed as we age or exercise.

Introduction:

The sympathetic nervous system is maintained in part by the neurotrophin, NGF (1-3). NGF has also been linked to maintenance of sensory neurons (4,5) and increases in NGF expression have been linked to increases in sympathetic and sensory innervation of target tissue (6,7). Thus NGF is an important regulator of both sympathetic, as well as sensory nervous system development, maintenance, innervation, and mapping of nerve fibers to target tissue.

Increases in NGF expression, in the vasculature, have been associated with hypertension (8,9). Hypertension is characterized by hypertrophy, hyperplasia and hyperinnervation of vascular smooth muscle cells in the resistance vessel walls (10,11). During development of hypertension, vascular smooth muscle cells undergo hyperplasia causing them to become hyperresponsive to neural stimulation (12). In spontaneously hypertensive rats (SHR) increased levels of NGF expression have been linked to hyperinnervation of the vasculature(4,13,14). Thus our main hypothesis is that increases in sympathetic innervation, as seen in the SHR, may lead to increased vasoconstriction causing a feedback loop that stimulates a further increase in blood pressure.

The sympathetic nervous system also plays an important role in controlling venous capacitance (15). In the deoxycorticosterone acetate (DOCA)-salt model of hypertension, it has been shown that there is increased venous tone (16) in hypertensive rats. Although most studies examining hypertension focus on arterial blood flow, venous regulation may play an important role in maintenance of systemic blood flow. Since veins carry about 70% of the body's blood volume, a slight shift or imbalance in sympathetic innervation may cause a shift in pressure homeostasis. This would translate into an increase in cardiac workload and stroke volume, which will lead to an increase in blood pressure (17). Studies conducted in SHR have also shown that venous tone is increased in hypertension (18). Thus changes in venous capacitance can have profound implications and effects on blood pressure regulation and overall peripheral resistance.

Exercise training has been shown to have beneficial effects on sympathetic nervous system function and overall cardiovascular health (19-21). We have shown exercise training to increase neurotrophic factor expression in skeletal muscle (22) and cardiac muscle (23,24). The current study was undertaken to determine whether increased physical activity affects neurotrophic factor expression in vascular tissue. If exercise alters vascular neurotrophic factor expression, then this could be responsible for changes in the sympathetic nervous system function observed with exercise. For these studies NGF protein expression, blood pressure and blood vessel

innervation were examined in 8 week old sedentary, 22 week old sedentary and 22 week old exercised rats.

Materials and Methods:

Subjects:

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. Twenty four Fisher 344 rats (Charles River, Portage MI), were used. These animals are the same animals that were used for the GDNF companion study. Eight animals were sacrificed at 8 weeks of age (8-wk-Sed), 8 were maintained for an additional 14 weeks without access to running wheels (22-wk-Sed) and 8 were maintained for an additional 14 weeks with access to running wheels (22-wk-VT). Animals were housed singly in Nalgene® cages with access to food and water *ad libitum*. Animals were kept on a 12:12 hour light-dark cycle in a room where the temperature was regulated (22-24°C). All animals were monitored daily and body weights were measured weekly to ensure positive weight gain.

General Tissue Collection and Processing for Measurement of NGF Content:

Animals were sacrificed via CO₂ asphyxiation followed by thoracotomy. All mesenteric arteries and veins were removed and cleaned of any fat and connective tissue. Vessels were either flash frozen by contact with dry ice and stored at -80°C for later determination of NGF content or placed in Zamboni's fixative and kept at 4°C prior to immunohistochemical processing.

Tissue Collection and Processing:

Just prior to processing, frozen vessel samples were dipped into liquid nitrogen and then crushed on a metal block chilled on dry ice. The pulverized vessel was then 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], 0.2 mM Benzamidine, 0.01 mM Benzethonium Chloride, and 0.2 mM ethylenediaminetetra-acetic acid (EDTA). The suspension was chilled on wet ice while being homogenized for 30 seconds using a variable speed Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). Homogenate was centrifuged at 13,000xg and the supernatant was analyzed using an enzyme-linked immunosorbant assay (ELISA) specific for NGF.

Enzyme-Linked Immunosorbant Assay (ELISA) for NGF:

Ninety six well plates (NUNC-Immuno™, Rochester, NY) were coated with 50 µl/well of 0.25 µg/ml anti-NGF monoclonal antibody (Chemicon, Temecula, CA) in 50 mM sodium carbonate buffer, pH 9.6, for 2 hours, at 37°C. Remaining sites on the plate were blocked with 125 µl/well of 1.0% BSA in carbonate buffer, for 1 hour, at 37°C. Plates were washed 3 times for 5 minutes with 250 µl/well of wash buffer (0.4 M NaCl, 0.1% Triton X-100 in 0.1 M phosphate buffer, pH 7.4). Following the third wash, 50 µl/well of NGF standard or samples in sample buffer were added. Each sample or standard was assayed in quadruplicate. Plates containing sample and NGF standard were incubated overnight, at room temperature, in a humidified chamber. The following day, plates were washed 3 times for 20 minutes with 250 µl/well of wash buffer. Each well then received 50 µl of wash buffer containing 1% BSA and anti-NGF secondary antibody conjugated to β-galactosidase (Chemicon, Temecula, CA), at a concentration of 10 mU/ml. After addition of anti-NGF secondary antibody conjugated to β-galactosidase, plates were placed in a humidified chamber and incubated at 37°C for 2 hours. Unbound secondary antibody was removed by washing plates 1 time for 1 minute and 2 times for 10 minutes with 250 µl/well of wash buffer with intermittent agitation. During the final wash, the galactosidase substrate chlorophenolred-beta-D-galactopyranoside (CPRG, Roche, Palo Alto CA) was dissolved in substrate buffer consisting of 0.1 M sodium phosphate and 1 mM MgCl₂ with 1% BSA at pH 7.4. Plates were incubated in substrate buffer, with CPRG,

overnight, at room temperature, in a humidified chamber. Absorbance was read at 575 nm on a microplate spectrophotometer.

Immunohistochemistry in Whole Mount Blood Vessels:

Mesenteric arteries and veins were removed and cleaned of any fat or connective tissue. The vessels were fixed overnight in Zamboni's fixative solution (2% Paraformaldehyde, 0.15% Picric Acid, in 0.1 M Phosphate Buffer) at 25°C. The tissue was washed 3 times for 10 minutes in dimethylsulfoxide. The tissue was then washed 3 times for 10 minutes in PBS. For NGF experiments, the tissue was blocked for an hour at 37°C in 10% mouse serum diluted with PBS containing 1% BSA. The tissue was incubated overnight at 25°C in primary antibody (Rabbit anti-NGF; 1:1000 dilution, Chemicon, Temecula, CA) in PBS-BSA 1%. The tissue was then washed 3 times for 5 minutes in PBS. Tissue was incubated for an hour at 37°C in secondary antibody (Mouse anti-Rabbit IgG biotin conjugate; 1:1500 dilution, Sigma, St. Louis, MO) in BSA-PBS 1%. The tissue was washed 3 times for 5 minutes in PBS. The tissue was incubated 30 minutes in PBS-BSA 1% containing fluorophore (streptavidin conjugated to Alexa Fluor 488 or 594; 1:1000 dilution, Molecular Probes, Eugene, OR). The tissue was washed 3 times for 5 minutes in PBS. The tissue was coverslipped, stored in 50% PBS-Glycerol at 4°C and was visualized on a confocal microscope. Controls for immunohistochemical analysis consisted of omission of anti-NGF primary antibody and also the use of non-immune rabbit serum as the primary antibody to identify any non-specific staining. Vessels were also stained for the following markers: calcitonin gene related peptide ((CGRP) Rabbit anti-CGRP; 1:8000 dilution in PBS-BSA 1%, Sigma, St. Louis, MO) and Tyrosine Hydroxylase ((TH) Rabbit anti-TH; 1:500 dilution in PBS-BSA 1%, Chemicon, Temecula, CA). Following an overnight wash, the second marker was applied and staining proceeded as previously stated. The tissue was coverslipped and visualized on a confocal microscope. Filter settings for Alexa Fluor 488 were band pass 505-530 and Alexa Fluor 594 were band pass 585-615. Samples were optically sectioned in 2 µm slices,

20 slices/image, and then optically reconstructed via LSM® software to attain a projection encompassing all layers of the vessel.

Blood Pressure Measurements:

Systolic blood pressure was measured using tail-cuff plethysmography (IITC. Inc., Woodland Hills, CA). Blood pressure information from the amplifier was recorded on a chart recorder (Gould Instrument Systems Inc., Dayton, OH). Animals were restrained in tubes in a heated chamber (29°C) and given 30 minutes to acclimate prior to blood pressure measurement.

Neurite Density Analysis:

Changes in neurite density were determined by placing a grid over each blood vessel image, as captured by the LSM software, and then counting the number of times neurites crossed the grid (25).

Statistical Analysis:

Comparisons between 8-wk-Sed, 22-wk-Sed and 22-wk-VT animals were done using an analysis of variance followed by a least significant difference (LSD) post hoc analysis. For all tests significance was set to $p < 0.05$. All data values are reported as the mean \pm standard error of the mean.

Results:

Gross Observations:

Body weights of 8-wk-Sed animals (170.3 ± 2.9 g) were significantly different from the 22-wk-Sed (274.8 ± 3.8 g) and 22-wk-VT animals (260.4 ± 4.5 g), $p < 0.05$. All animals showed positive weight gain throughout the study. Following tissue removal, it was noted that the mesenteric vessels from 8-wk-Sed and 22-wk-VT animals had a very healthy and lean appearance in contrast to vessels from the 22-wk-Sed animals, which had large deposits of fat and connective tissue around them.

Distances Run:

For the voluntary running studies, 8-week-old rats were introduced to cages with running wheels. The average distance run by the rats steadily increased over a 5 week time period, stabilizing at 250 ± 75 m/day (1.7 ± 0.5 km/week = 1.1 ± 0.3 miles per week).

Blood Pressure:

Eight week old sedentary rats had a systolic blood pressure of 129.6 ± 3.2 mm Hg. By 22 weeks of age systolic blood pressure in sedentary rats was significantly elevated (146.7 ± 1.9 mm Hg) compared to that in 8-week-old sedentary rats. Fourteen weeks of voluntary exercise caused a significant decrease in systolic blood pressure (124.14 ± 3.2 mmHg) compared to that in age matched 22-wk-sedentary rats, $p < 0.05$.

NGF Protein Content in Mesenteric Vessels:

NGF protein content in arteries (1.14 ± 0.22 pg NGF/vessel; $n = 6$) and veins (1.2 ± 0.31 pg NGF/vessel; $n = 4$) from 8-wk-Sed animals was significantly higher than that in arteries (0.34 ± 0.10 pg NGF/vessel; $n = 6$) and veins (0.25 ± 0.03 pg NGF/vessel; $n = 7$) from 22-wk-Sed animals (Figure 1). NGF protein content in arteries (0.92 ± 0.16 ; $n = 8$) and veins (0.42 ± 0.07 ; $n = 8$) from 22-wk-VT animals was significantly greater than that in 22-wk-Sed animals. NGF protein content was not significantly different in the mesenteric arteries and veins of 22-wk-VT compared to 8-wk-Sed animals (Figure 1), $p < 0.05$.

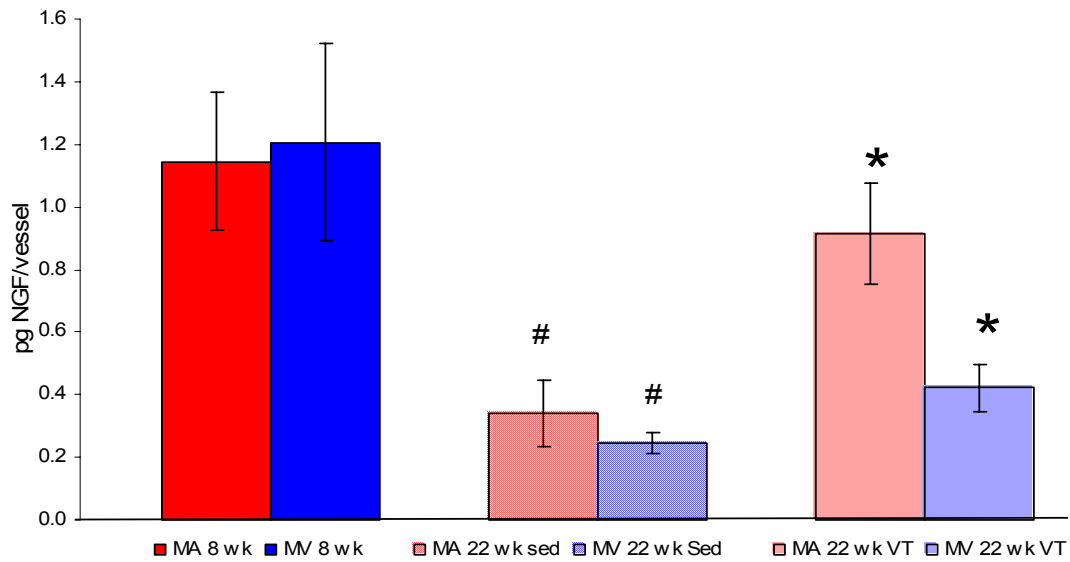


Figure 1: NGF Protein Content in Mesenteric Arteries and Veins. Mesenteric arteries (MA) and veins (MV) were removed from 8-week-old sedentary (8-wk-Sed; n = 4-6), 22-week-old sedentary (22-wk-Sed; n = 6-7) and 22-week-old rats following 14 weeks of voluntary running (22-wk-VT; n=8), and NGF protein content was determined by ELISA. NGF protein content of mesenteric vessels from sedentary rats declined significantly between 8 and 22-weeks of age. Following 14 weeks of voluntary running, NGF protein content was found to be significantly elevated (*) in mesenteric arteries and veins compared to levels in age-matched sedentary controls (p<0.05, ANOVA, LSD).

NGF Localization in Vessels from 8 and 22 Wk Old Rats:

Immunohistochemical studies were done to test which type of nerves contained NGF. Sympathetic nerve fibers were identified using antibodies against Tyrosine Hydroxylase and sensory nerve fibers were identified using antibodies against Calcitonin Gene Related Peptide.

Vessels from 8 Week Old Sedentary Rats:

The vessels from 8-wk-Sed (Figure 2, 3) all showed fibers staining positive for NGF, CGRP and TH. In vessels from 8-wk-Sed rats, NGF and TH were both present in arteries and veins, but did not appear to be colocalized in the same fibers (Figure 3A). NGF and CGRP were present in both arteries and veins, and appeared to be colocalized in the same fibers (Figures 2A). In both arteries and veins, fibers staining positive for TH and CGRP were in close proximity to one another, but these antigens were never colocalized in the same fibers (data not shown).

Vessels from 22-week-old Sed animals showed fibers staining positive for NGF, CGRP and TH. In contrast to the 8-week-old Sed, NGF and TH, although still present in both arteries and veins, now appeared to be colocalized to the same fibers (Figure 3B). Also in contrast to the localization seen in 8-wk Sed and 22-wk VT animals, NGF and CGRP, although still present in both vessels, now appeared to be localized to different fibers (Figure 2B). In both arteries and veins, fibers staining positive for TH and CGRP were in close proximity to one another, but these antigens were never colocalized to the same fibers (data not shown).

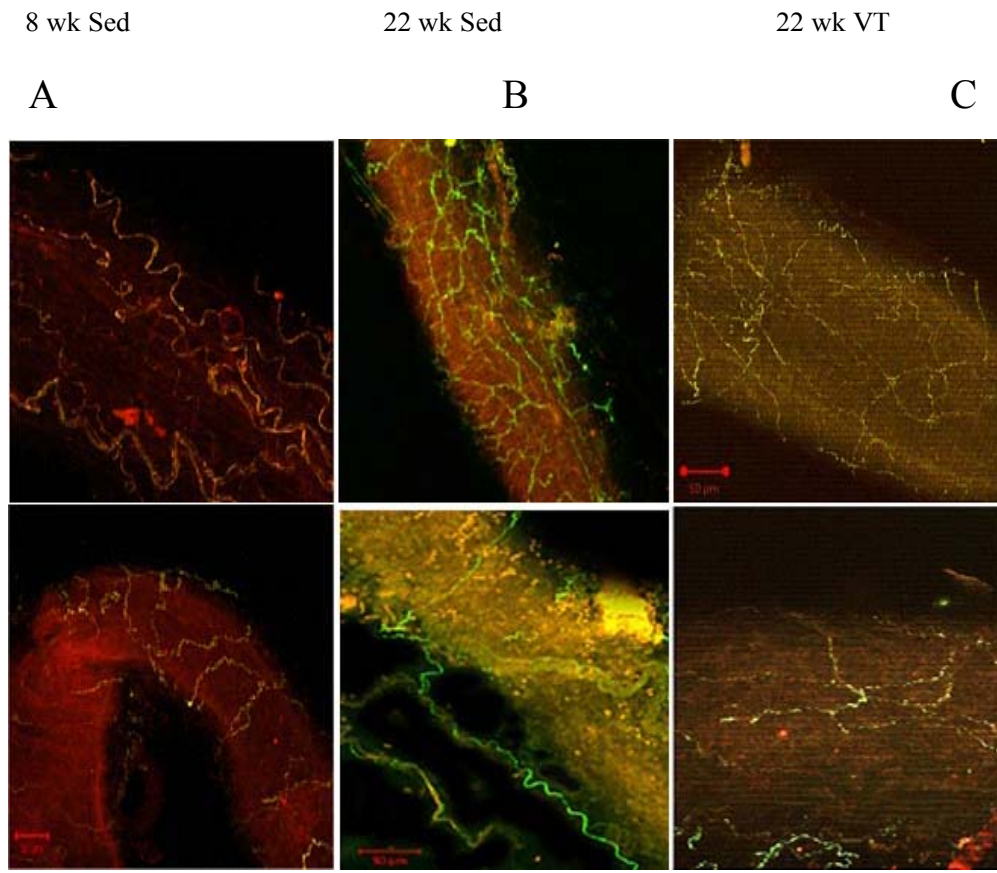


Figure 2: Immunohistochemical Staining of NGF Positive and CGRP Positive Fibers in Mesenteric Arteries and Veins. Mesenteric arteries and veins were removed from 8-week-old sedentary (8-wk-Sed), 22-week-old sedentary (22-wk-Sed) and 22-week-old rats following 14 weeks of voluntary running (22-wk-VT), and processed for immunohistochemical staining. The figure displays mesenteric arteries (top panels) and mesenteric veins (bottom panels) were reacted with antibodies against the sensory neurotransmitter calcitonin gene related peptide (CGRP; green) and nerve growth factor (NGF; red). In vessels from 8-week-old sedentary rats (Left panels) and 22-week-old exercised rats (Right panels), NGF and CGRP were found to be colocalized to the same fibers (appear as yellow), while in vessels from 22-week-old sedentary rats (Center panels) NGF and CGRP were not found in the same fibers.

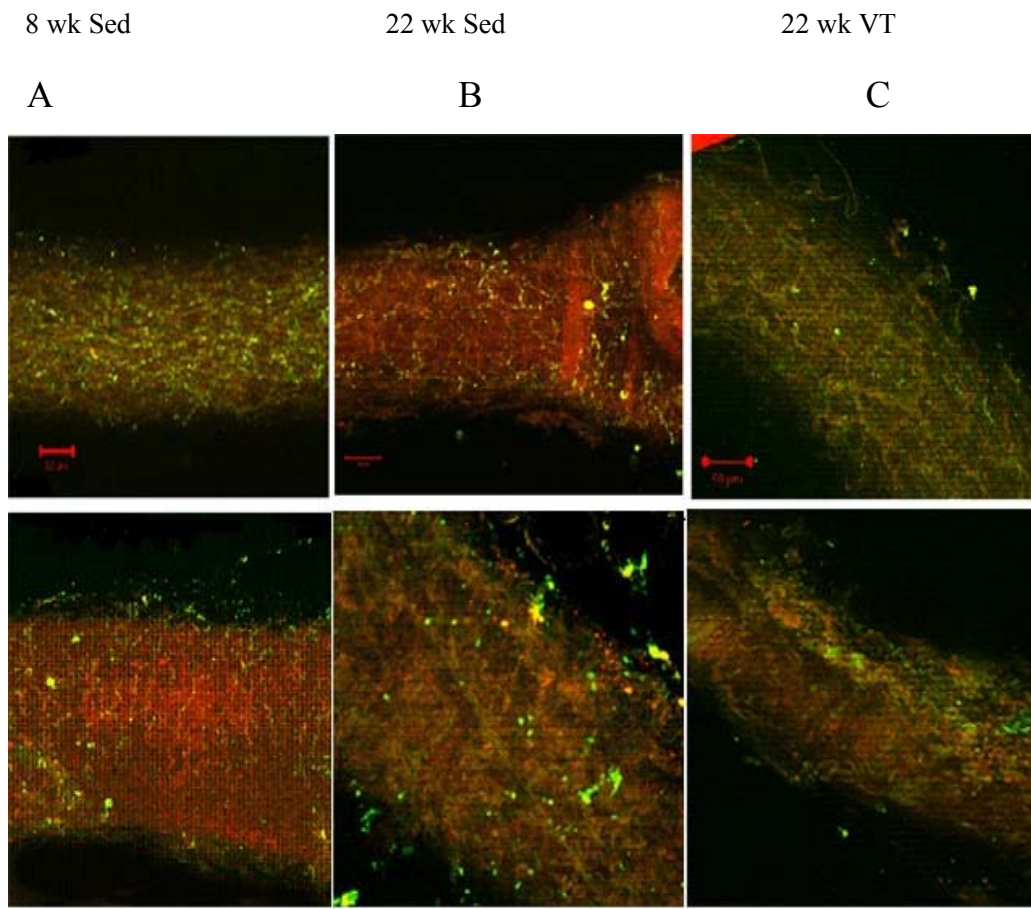


Figure 3: Immunohistochemical Staining of NGF Positive and TH Positive Fibers in Mesenteric Arteries and Veins. Mesenteric arteries and veins were removed from 8-week-old sedentary (8-wk-Sed), 22-week-old sedentary (22-wk-Sed) and 22-week-old rats following 14 weeks of voluntary running (22-wk-VT), and processed for immunohistochemical staining. The figure displays mesenteric arteries (top panels) and mesenteric veins (bottom panels) were reacted with antibodies against tyrosine hydroxylase (TH; green) a marker for sympathetic neurons, and nerve growth factor (NGF; red). In vessels from 8-week-old sedentary rats (Left panels) and 22-week-old exercised rats (Right panels), NGF and TH were not found in the same fibers, while in vessels from 22-week-old sedentary rats (Center panels) NGF and TH were colocalized to the same fibers(appear as yellow).

Vessels from 22-week-old VT animals showed fibers staining positive for NGF, CGRP and TH. In 22-wk-old VT rats NGF and TH were both present in arteries and veins, but did not appear to be localized to the same fibers (Figures 3C). NGF and CGRP were present in both arteries and veins, and appeared to be colocalized to the same fibers (Figures 2C). In both arteries and veins, fibers staining positive for TH and CGRP were in close proximity to one another, but these antigens were never colocalized to the same fibers (data not shown).

Nerve Fiber Density:

Nerve fiber density was assessed in the mesenteric arteries and veins. In the mesenteric arteries from sedentary animals, the density of NGF- and TH-positive fibers increased significantly between 8 and 22-weeks of age (Figure 4). Following 14 weeks of exercise, there was a significant decrease in the number of NGF positive and TH positive fibers, as well as a significant increase in the number of CGRP positive fibers compared to arteries from age matched sedentary controls (Figure 4). In the mesenteric veins from sedentary animals, the density of NGF positive fibers increased significantly between 8 and 22-weeks of age in sedentary animals. Following 14 weeks of exercise, there was a significant decrease in the density of NGF- and TH-positive fibers and a significant increase in the density of CGRP positive fibers compared to veins from age-matched sedentary controls (Figure 4).

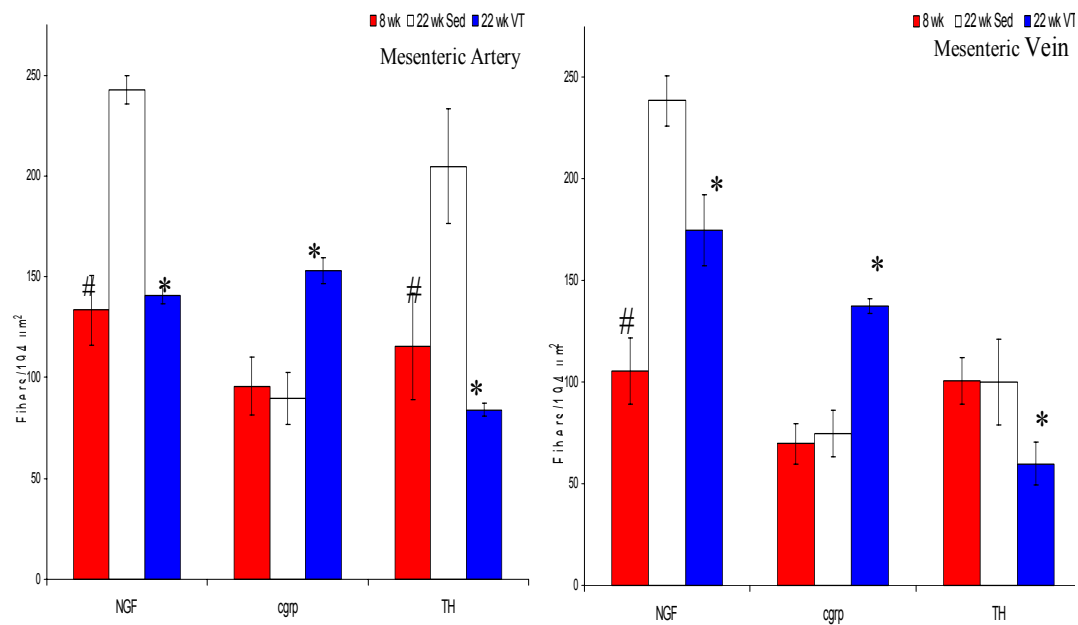


Figure 4: Density of Nerve Fibers in the Walls of Mesenteric Arteries and Veins. Mesenteric arteries and veins were removed from 8-week-old sedentary (8-wk-Sed), 22-week-old sedentary (22-wk-Sed) and 22-week-old rats following 14 weeks of voluntary running (22-wk-VT), and processed for immunohistochemical staining. Mesenteric arteries (Left Panel) and mesenteric veins (Right panels) were reacted with antibodies against tyrosine Hydroxylase (TH), a marker for sympathetic neurons, calcitonin gene related peptide (CGRP), a sensory neurotransmitter, and nerve growth factor (NGF). The density of NGF-, TH-, and CGRP-positive fibers was counted by placing a grid over images ($194 \mu\text{m}^2/\text{image}$) of vessels, and counting the number times that nerve fibers crossed the grid. In mesenteric arteries, from sedentary rats, the density of NGF-positive and TH-positive fibers increased significantly (#) between 8 and 22 weeks of age. Following 14 weeks of exercise, there was a significant decrease in NGF-positive and TH-positive fibers as well as a significant increase in CGRP-positive fibers compared to that in vessels from age-matched sedentary animals (*). In the mesenteric veins from sedentary rats, the density of NGF-positive fibers increased significantly between 8 and 22-weeks of age. Following 14 weeks of exercise, there was a significant decrease in NGF- and TH- positive fibers, as well as a significant increase in CGRP- positive fibers compared to that in vessels from age-matched sedentary controls.

Discussion:

In the current study, we have demonstrated novel findings concerning NGF expression and sympathetic and sensory innervation in the mesenteric vascular bed. The NGF protein content of mesenteric vessels decreases between 8 and 22-weeks of age in sedentary rats. During this same time blood pressure increased. Voluntary exercise resulted in a lowering of blood pressure and restored the NGF protein content of mesenteric vessels to levels similar to those seen in 8 week old animals. In mesenteric vessels from 22-week-old rats, NGF was found to colocalize to TH, a marker of sympathetic innervation. While in vessels from 8-week-old sedentary rats and 22-week-old exercised rats NGF was found to be colocalized to CGRP, a marker for sensory innervation. Taken together, these data suggest that as we age and move toward a phenotype more consistent with hypertension, innervation patterns may shift from a balanced sensory/sympathetic innervation pattern seen in the younger animals to a predominantly sympathetic innervation pattern in older animals.

It has been well established that NGF expression in dorsal root ganglion, middle cerebral arteries, basal forebrain neurons, hippocampal neurons, spinal motoneurons and aorta decreases with age(26-31). Nerve fiber loss in cerebral blood vessels (27) and decreased nerve regeneration have been shown with increased age (32). In the current study we have shown that NGF protein content in the mesenteric artery and vein decreases between 8 and 22 weeks of age. This decrease in NGF expression may have implications for survival of neurons and neural plasticity. This decrease may be responsible for the decline in neurotrophic factor in peripheral ganglia and may help account for neuronal atrophy that is observed with aging.

Although neurons require neurotrophic factors for development, as the neurons age the dependency on neurotrophic factors may change. In a study conducted with IB4-binding neurons, neurons switched from dependence on NGF in embryonic life to dependence on GDNF in postnatal life (33). Embryonic mouse trigeminal ganglion switch from dependency on brain derived neurotrophic factor as an embryo to dependency later in life on NGF (34). Switches in dependency from other tropic

factors to NGF in sensory neurons have also been shown (33-38). Full phenotypic differentiation of sensory neurons requires NGF/TrkA signaling to regulate peripheral target field innervation (39). Our data showing NGF colocalization with CGRP positive fibers at 8 weeks, colocalization with TH positive fibers in 22 week old sedentary animals and then again with CGRP positive fibers in 22 week old exercised animals, suggests that the sensory fibers continue to require NGF in this vascular bed. Furthermore, the data suggest that decreases in target derived trophic factor may be a critical event in the aging process of sensory and sympathetic neurons.

Results of our study illustrate the potential beneficial effects of exercise on the nervous system. Exercise has been shown to upregulate trophic content in rat hippocampal neurons in aged animals, back to levels which parallel those seen in younger rats (40). We have demonstrated that NGF protein content is higher in the vessels of 22-week-old exercised animals compared to vessels from 22-week-old sedentary animals. We also showed that the density of fibers staining positive for both NGF and CGRP was significantly greater in vessels from exercised animals versus age-matched sedentary animals, and that blood pressures of the exercised animals were significantly lower than that in the sedentary age-matched controls. Here we show parallels in our data to several studies conducted in which old vessels were reinnervated close to the younger animal patterns with infusions of NGF (41-43) as well as ones demonstrating that NGF can reverse alterations in structure to neurons which have undergone transformations due to aging(4,26,44). Although the concentration of NGF/vessel decreased between 8-week-sed and 22-week sed, the innervation density increases. This may be due to an overall increase in retrograde transport. As the density of nerve fibers increases with sedentary behavior, the amount of target derived trophic factor may increase. It may follow that the increase in the amount of fibers present may cause increased shipping of trophic factor to the nerve cell body. Conversely, as the animals exercise, the density of innervation decreases but the amount of NGF protein increases. This may also be accounted for by decreases in retrograde transport. Although there is increased protein available the

number of fibers available decreased and therefore the amount of protein present in or at the vasculature will be higher than that in sedentary animals.

Results of studies on spontaneously hypertensive rats provide good evidence that alterations in vascular NGF expression can lead to changes in sympathetic (45-47) and sensory nervous system function(48). Calcitonin gene-related peptide (CGRP), a peptide neurotransmitter found in sensory neurons, has been shown to be a potent vasodilator (49). CGRP can be released from sensory nerve endings following a variety of stimuli, including exposure to capsaicin, with antidromic stimulation (50) or with changes in hydrogen ion content of tissues (51). It is interesting that Supowit *et al.*(48) reported a decrease in CGRP content in sensory neurons in SHR, despite the fact that NGF levels are elevated in vascular tissues. A decrease in the level of expression of CGRP, a vascular relaxant, would help to drive blood pressure higher in SHRs. Our data supports these findings by showing a decrease in CGRP positive fibers with aging sedentary behavior, an increase in TH positive fibers, and an increase in blood pressure.

We propose that there may be a shift from balanced sensory/sympathetic innervation pattern in mesenteric vessels early in life to a predominantly sympathetic pattern in older sedentary individuals and a reversal of this with exercise. This has great implications for helping to ameliorate conditions seen in the elderly or injured population. Instead of medication, beneficial effects to the nervous system may be gained by modifications in lifestyle. With a change in physical activity the amount of trophic factor content of the vessels may be increased returning the nervous system and vascular innervation back to a more sensory innervation pattern, while helping to lower blood pressure. Thus, as we age, increases in physical activity may reverse some of the damaging effects of aging by increasing target derived trophic factors which maintain the nervous system in a healthy and plastic state.

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INTRODUCTION TO CHAPTER IV

Following the studies determining the presence of GDNF in the vasculature and its changing role with exercise we chose to see what effect hypertension may have on GDNF expression in the vasculature. The objective of this study was to determine whether GDNF is increased with the development of hypertension and whether increased pressure may be the initiating factor which may cause the increase in trophic factor expression seen in hypertension. We hypothesize that GDNF levels in the mesenteric vessels will be lower in normotensive animals than in hypertensive animals in two well established models of hypertension, the genetic SS/JR model and the pharmacological L-nitroarginine methyl ester (LNAME) model.

Following six weeks of either a low salt (0.4% sodium) or high salt (4% sodium) diet eight SS/JR high salt and eight age matched controls were euthanized and mesenteric arteries and veins were removed. A second model of hypertension was used, the LNAME model using Fisher 344 rats. Both sets of vessels were analyzed for GDNF content by enzyme-linked immunosorbant assay and patterns of innervation and distribution of GDNF in mesenteric arteries and veins were examined using immunohistochemical methods. Results indicated that GDNF expression was increased with the development of hypertension versus the age matched normotensive controls.

In addition, *in vitro* studies were performed. Eight 5 week old and eight 12 week old Fisher 344 rats were euthanized and mesenteric vessels were hand dissected out and pressurized to either normotensive physiological pressures or hypertensive pressures corresponding to the vascular bed and the GDNF protein content was also assessed via ELISA.

GDNF levels as evaluated by ELISA were significantly higher in hypertensive animals both in the SS/JR model as well as the LNAME model. In addition, following 4 hours of elevated pressure the GDNF protein content in both 5 wk old and 12 wk old vessels pressurized to hypertensive pressures was significantly greater than the vessels held at normotensive pressures. The results indicate that as the animals developed hypertension the GDNF protein content increases and changes in

trophic factor expression may help to maintain or exacerbate the hypertensive state. Taken together these data suggest that GDNF can stimulate the growth of axons, both sympathetic and sensory, to target tissue supporting fiber outgrowth, which may lead to increased vasoconstriction causing a feedback loop that stimulates a further increase in blood pressure.

CHAPTER IV

GLIAL CELL LINE DERIVED NEUROTROPHIC FACTOR EXPRESSION IN THE MESENTERIC ARTERY AND VEIN IS INCREASED IN TWO MODELS OF HYPERTENSION

Abstract:

In the peripheral nervous system, Glial cell line-derived neurotrophic factor (GDNF) affects sensory, autonomic and somatic motor neurons. The structure and function of neurons innervating a given target tissue may be dependent on trophic factor expression by that tissue. Recent studies have shown that there are different populations of sympathetic neurons innervating mesenteric arteries and veins. However the role that neurotrophic factors play in hypertension and mesenteric vessels is not well understood. We hypothesize that GDNF levels in the mesenteric vessels will be lower in normotensive animals than in hypertensive animals in two well established models of hypertension, the genetic SS/JR model and the pharmacological LNAME model. The objective of this study was to determine whether GDNF is increased with the development of hypertension and whether increased pressure may be the initiating factor which may cause the increase in trophic factor expression seen in hypertension.

Following six weeks of either a low salt (0.4% sodium) or high salt (4% sodium) diet eight SS/JR high salt and eight age matched controls were euthanized and mesenteric arteries and veins were removed and analyzed for GDNF content by enzyme-linked immunosorbant assay. Patterns of innervation and distribution of GDNF in mesenteric arteries and veins were examined using immunohistochemical methods. Results indicated that GDNF expression was increased with the development of hypertension versus the age matched normotensive controls.

A second model of hypertension was used, the LNAME model using Fisher 344 rats. Eight animals were euthanized at 5 weeks and GDNF content by enzyme-linked immunosorbant assay. Patterns of innervation and distribution of GDNF in mesenteric arteries and veins were examined using immunohistochemical methods. A second set of mesenteric vessels were hand dissected out and pressurized to either normotensive physiological pressures or hypertensive pressures corresponding to the vascular bed and the GDNF protein content was also assessed. Following seven weeks of LNAME treatment eight hypertensive animals and eight untreated age matched controls were euthanized and GDNF content was assessed by enzyme-linked

immunosorbant assay and innervation patterns were assessed. A second set of mesenteric vessels were also hand dissected out and pressurized to either normotensive physiological pressures or hypertensive pressures corresponding to the vascular bed and the GDNF protein content was also assessed via ELISA.

GDNF levels as evaluated by ELISA were significantly higher in hypertensive animals both in the SS/JR model as well as the LNAME model. In addition, following 4 hours of elevated pressure the GDNF protein content in both 5 wk old and 12 wk old vessels pressurized to hypertensive pressures was significantly greater than the vessels held at normotensive pressures. The results indicate that as the animals developed hypertension the GDNF protein content increases and may help to maintain or exacerbate the hypertensive state.

Introduction:

Hypertension is characterized in spontaneously hypertensive rats (SHR) by hypertrophy, hyperplasia and hyperinnervation of vascular smooth muscle cells in the resistance vessel walls(1,2). During hypertension, smooth muscle cells undergo hyperplasia which may result in the vascular smooth muscle cells becoming more sensitive to increased neural stimulation, i.e. increased sympathetic innervation (3). Increased sympathetic innervation may lead to increased vasoconstriction of the vasculature possibly causing a feedback loop that stimulates a further increase in blood pressure. A variety of neurotrophic factors, including NGF and GDNF, have been implicated in the maintenance of neuronal cell bodies, proper innervation of the vascular wall (4), maintaining neural plasticity (5-9), as well as influencing nerve growth in vivo (10-12).

GDNF is important in the growth and preservation of central and peripheral neurons, is most prominently expressed in the gut, and is essential for the development of the enteric nervous system (13,14). GDNF also acts on receptors in autonomic neuronal populations and sympathetic ganglia, supporting fiber growth in peripheral ganglia (15,16). In addition to its role in the sympathetic nervous system, GDNF is also involved in control of parasympathetic innervation and in the

development of dorsal root sensory neurons (17). Studies conducted after dorsal root injury indicated that with chronic administration of GDNF, sensory axons were able to restore connections with their target tissue, restoring sensory function (18). Taken together these data suggest that GDNF can stimulate the growth of axons to target tissue supporting fiber outgrowth.

Infantile hypertrophic pyloric stenosis (IHPS) is a narrowing of the pylorus of the stomach caused by smooth muscle thickening. Studies conducted in IHPS indicate that smooth muscle hypertrophy any hyperplasia are associated with increased production of GDNF (19). As hypertrophy is a hallmark of hypertension, we propose that the smooth muscle cells lining the walls of the vasculature will increase their GDNF expression as they become hypertrophic. Overexpression of GDNF may alter structure and function in several neuronal populations that innervate the vasculature.

In addition to affecting arterial function, the sympathetic nervous system plays an important role in controlling venous capacitance (20). In previous studies conducted in our laboratory, GDNF colocalizes to smooth muscle cells, which in hypertension undergo hyperplasia. One would predict that tropic factor expression would increase and could possibly lead to increased sympathetic innervation and increased venous tone. In the deoxycorticosterone acetate (DOCA)-salt model of hypertension, it has been shown that there is increased venous tone in the resistance vasculature (21). Although most studies done on hypertension focus on arterial blood flow, venous regulation may play an important role in maintenance of systemic blood flow. Since veins carry about 70% of the body's blood volume, a slight shift or imbalance in sympathetic innervation may cause a shift in pressure homeostasis. This would translate into increase cardiac workload and stroke volume, which will lead to an increase in blood pressure (22). The sympathetic nervous system is an important regulator of venous capacitance (20). Studies conducted in SHR have also shown that venous tone is increased in hypertension (23). Thus changes in venous capacitance can have profound implications and effects on blood pressure regulation and overall cardiovascular function. Our model predicts that GDNF expression increases in veins and this change could lead to hyperinnervation of veins by sympathetic nervous

system. Increased sympathetic tone in veins may result in decreased capacitance and increased venous pressure.

In order to understand blood pressure regulation, the mechanisms regulating innervation of the smooth muscle in the vasculature is paramount. Understanding the effects of increased or decreased expression of neurotrophic factors by vascular smooth muscle and changes in innervation in the vascular beds helps to increase our knowledge of the basic neural regulation of blood vessel tone. Insight into neurotrophic factor expression in the vasculature is important because neuronal control of resistance vessels exemplifies one of the major controlling factors for regulation of blood pressure. In addition to gaining knowledge into some of the basic differences of how arteries and veins regulate blood flow, results of this study may provide some answers into the mechanism of hypertension development.

Materials and Methods:

Subjects:

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. Sixteen Dahl Salt-sensitive rats (SS/JR) (Harlan, Indianapolis, IN) and twenty-four Fisher 344 rats (F344) (Charles River, Portage, MI) were used. SS/JR rats were divided into a low salt group (n = 8; LS), and a high salt group (n = 8; HS). F344 rats were divided into three groups; young animals (n = 8; 5 wk), normotensive animals (n = 8; NT), and hypertensive (n = 8; HTN). Fisher 344 rats in the hypertensive group received 10mg/L of L-nitroarginine methyl ester (LNAME, Sigma, St. Louis, MO) in their drinking water for 7 weeks. Water consumption was monitored daily. No significant difference in water consumption was noted among animals consuming LNAME. All animals participated in normal ambulation. Animals were housed one per cage in Nalgene® cages with access to food and water *ad libitum*. Animals were kept on a 12:12 hour light-dark cycle in a room where the

temperature was regulated (22-24°C). All animals were monitored daily and body weights were measured weekly to ensure positive weight gain.

General Tissue Collection:

Animals were sacrificed via CO₂ asphyxiation followed by thoracotomy. All mesenteric arteries and veins were removed and cleaned of any fat and connective tissue. Vessels were either flash frozen by contact with dry ice and stored at -80°C for later determination of GDNF content or placed in Zamboni's fixative and kept at 4°C prior to immunohistochemical processing(See below).

Tissue Processing:

Just prior to processing, frozen vessel samples were dipped into liquid nitrogen and then crushed on a metal block chilled on dry ice. The pulverized vessel was then suspended in 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, 1.64 µl/1 mL, Sigma], 0.2 mM Benzamidine, 0.01 mM Benzethonium Chloride, and 0.2mM ethylenediaminetetraacetic acid (EDTA)). The suspension was chilled on wet ice while being homogenized for 30 seconds using a variable speed Tissue Tearor (Biospec Products, Inc.). Homogenate was centrifuged at 13,000xg and the supernatant was analyzed using an enzyme-linked immunosorbant assay (ELISA) specific for GDNF.

Enzyme-Linked Immunosorbant Assay (ELISA) for GDNF:

Ninety six well plates (NUNC-Immuno™, Rochester, NY) were coated with 100 µl/well of 1 µg/ml anti-GDNF monoclonal antibody (R&D Systems, Minneapolis, MN) in 0.1 M phosphate buffer (PBS), pH 7.4, overnight at room temperature (RT) in a humidified chamber. Plates were washed 2 times 1 minute with 250 µl/well of wash buffer containing 0.4 M NaCl and 0.05% Tween-20 in 0.1 M PBS, pH 7.4. Remaining sites on the plate were blocked with 200 µl/well of 1.0% bovine serum albumin (BSA), 5% sucrose in PBS for 1 hour at RT in a humidified

chamber. Plates were washed 3 times. Following the third wash, 100 µl/well of GDNF standard or sample was added. Fresh standards were made for each assay and were diluted in sample buffer (wash buffer containing 2 mM EDTA and 0.5% BSA). Each sample and standard were assayed in quadruplicate. Plates containing sample and GDNF standard were incubated for 2 hours at RT in a humidified chamber. The plates were washed. Each well then received 100 µl of biotinylated anti-GDNF 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 were washed 3 times. Each well then received 100 µl of streptavidin linked β-galactosidase (100 µg/ml, Molecular Probes, Eugene, OR) diluted in TBS containing 1% BSA and 0.05% Tween-20 and were placed in a humidified chamber and incubated at RT for 20 minutes. The plates were washed. During the final wash, the galactosidase substrate chlorophenolred-beta-D-galactopyranoside ((CPRG), Roche, Indianapolis, IN) was dissolved in substrate buffer consisting of 0.1 M sodium phosphate and 1 mM MgCl₂ at pH 7.4 with 1% BSA. Plates were incubated in substrate buffer with CPRG overnight at room temperature in a humidified chamber. Absorbance was measured at 575 using a microplate spectrophotometer.

Immunohistochemistry in Whole Mount Blood Vessels:

Mesenteric arteries and veins were removed and cleaned of any fat or connective tissue. The vessels were fixed overnight in Zamboni's fixative solution (2% Paraformaldehyde, 0.15% Picric Acid, in 0.1 M Phosphate Buffer) at 25°C. The tissue was washed 3 times for 10 minutes in dimethylsulfoxide. The tissue was then washed 3 times for 10 minutes in PBS. For GDNF, the tissue was blocked for an hour at 37° C in 10% mouse serum diluted with PBS-BSA 1%. The tissue was incubated overnight at 25 ° C in primary antibody (Rabbit anti-GDNF; 1:500 dilution Santa Cruz, Santa Cruz, CA) in PBS-BSA 1%. The tissue was then washed 3 times for 5 minutes in PBS. Tissue was incubated for an hour at 37 ° C in secondary antibody (Mouse anti-Rabbit IgG biotin conjugate; 1:1500 dilution, Sigma, St. Louis,

MO) in PBS-BSA 1 %. The tissue was washed 3 times for 5 minutes in PBS. The tissue was incubated 30 minutes in PBS-BSA 1% containing fluorophore (streptavidin conjugated to Alexa Fluor 488 or 594; 1:1000 dilution, Molecular Probes, Eugene, OR). The tissue was washed 3 times for 5 minutes in PBS. The tissue was coverslipped, stored in 50% PBS-Glycerol at 4°C and was visualized on a confocal microscope. Controls for immunohistochemical analysis consisted of omission of anti-GDNF primary control and also the use of non-immune rabbit serum as the primary antibody to identify any non-specific staining. Vessels were also stained for the following markers: calcitonin gene related peptide ((CGRP)Rabbit anti-CGRP; 1:8000 dilution in PBS-BSA 1%, Sigma, St. Louis, MO) and Tyrosine Hydroxylase ((TH) Rabbit anti-TH; 1:500 dilution in PBS-BSA 1%, Chemicon, Temecula, CA). Following an overnight wash, the second marker was applied and staining proceeded as previously stated. The tissue was coverslipped and visualized on a confocal microscope. Filter settings for Alexa Fluor 488 were band pass 505-530 and Alexa Fluor 594 were band pass 585-615. Samples were optically sectioned in 2 µm slices, 20 slices/image, and then optically reconstructed via LSM® software to attain a projection encompassing all layers of the vessel.

Isolated Cannulated Vessel Experiments:

First-order mesenteric arteries or veins were isolated by microdissection. Tissue was removed and placed in a cooled (4 ° C) dissection chamber filled with physiological salt solution (PSS) with the following composition in mM: NaCl 130; KCl 4.7; MgCl₂ 2.4; MOPS 2; glucose 1; .1 Na₂EDTA; pH 7.4, 295-300 mOsm and supplemented with 0.03 mM pyruvic acid. Vessels were cleaned of any fat and connective tissue. A 1-2 mm unbranched section of vessel was hand dissected out using a stereomicroscope and was transferred to a lexan cannulation chamber containing Ca⁺² free PSS 0.1% BSA solution and equipped with glass micropipette micromanipulators on both ends. Arteries or veins were cannulated at both ends with glass micropipettes (~110µm tip). The vessel ends were tied onto the glass pipettes with 11-0 monofilament suture. Vessels were slowly warmed to 37°C and superfused

with PSS containing 1.8 mM CaCl₂. Vessels were initially pressurized (100 mm Hg for arteries; 20 mm Hg mm for veins) to equilibrate the tissue and to test for any leaks. Once the vessel was equilibrated for 20 minutes the pressure was lowered to 50 mm Hg for arteries (average physiological pressure) or 4 mm Hg for veins and held for four hours. Another group of vessels was pressurized as described above, but after reaching equilibration, the pressure was adjusted to 100 mm Hg (hypertensive pressure for arteries) or 15 mm Hg for veins (hypertensive pressure for veins) and held for four hours. At the completion of each experiment vessels were snap frozen on dry ice prior to proceeding to the ELISA protocol for GDNF protein analysis.

Blood Pressure Measurements:

Systolic blood pressure was measured using tail-cuff plethysmography (IITC. Inc., Woodland Hills, CA). Blood pressure information from the amplifier was recorded on a chart recorder (Gould Instrument Systems Inc., Dayton, OH). Animals were restrained in tubes in a heated chamber (29°C) and given 30 minutes to acclimate prior to blood pressure measurement.

Neurite Density Analysis:

Changes in neurite density were determined by placing a grid over each blood vessel image, as captured by the LSM software, and then counting the number of times neurites crossed the grid (24).

Statistical Analysis:

Comparisons between each group, in their respective studies, were done using an analysis of variance followed by a least significant difference (LSD) post hoc analysis. For all tests significance was set to $p < 0.05$. All data values are reported as the mean \pm standard error of the mean.

Results:

Gross Observations:

Body weights of SS/JR LS animals (437.3 ± 6.3 g) HS (422.5 ± 10.4 g) were not significantly different from one another. Body weights for the Fisher animals were 5 wk (129.37 ± 4.4 g), NTN (323.0 ± 3.32 g), HTN (309.87 ± 4.09 g). There was no statistical difference in weight between normotensive or hypertensive animals. All animals showed positive weight gain throughout the study.

Blood Pressure Measurements:

SS/JR LS rats had a significantly lower systolic blood pressure of 164 ± 9 mm Hg compared to the SS/JR HS rats (234 ± 18 mm Hg). The control rats used in the LNAME study had a significantly higher systolic blood pressure of 143 ± 5 mm Hg versus the control rats (119 ± 3 mm Hg).

ELISA Analysis:

GDNF Protein Content in Mesenteric Vessels : SS/JR Rats:

GDNF protein content in LS arteries (0.929 ± 0.06 pg GDNF/mg tissue; n = 8) and veins (4.914 ± 0.22 pg GDNF/mg tissue; n = 8) was significantly different than HS arteries (1.432 ± 0.219 pg GDNF/mg tissue; n = 6) and HS veins (6.439 ± 0.50 pg GDNF/mg tissue; n = 6) (Figure 1).

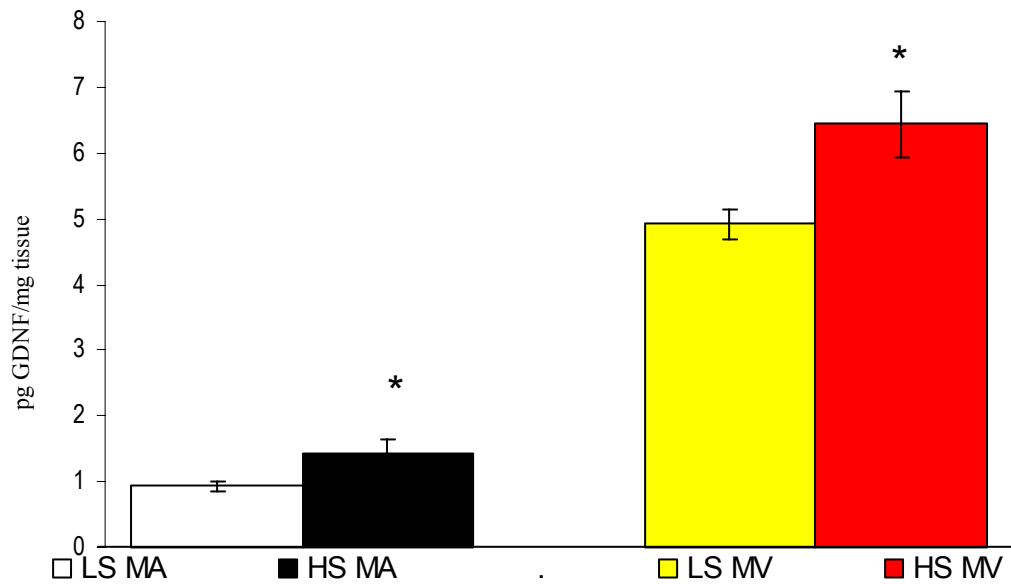


Figure 1: GDNF Protein Content in Mesenteric Arteries and Veins from SS/JR Rats. Mesenteric arteries (MA) and veins (MV) were removed from LS (n = 8) and HS (n = 8) animals and GDNF protein content was determined by ELISA. GDNF protein content of mesenteric vessels from HS rats was significantly increased compared to vessels from LS animals.

GDNF Protein Content in Mesenteric Vessels: LNAME Rats-5 Weeks:

GDNF protein content in NTN arteries (2.61 ± 0.66 pg GDNF/mg tissue; n = 8) was significantly lower than in the veins (5.72 ± 0.58 pg GDNF/mg tissue; n = 8)(Figure 2).

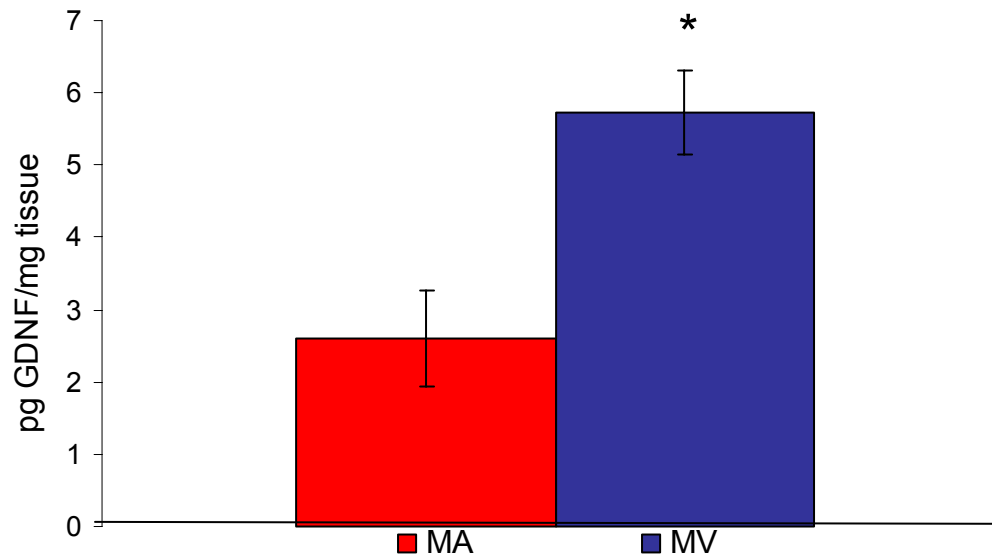


Figure 2: GDNF Protein Content in Mesenteric Arteries and Veins from 5 Wk Old Untreated Fisher 344 Rats. Mesenteric arteries (MA) and veins (MV) were removed from 5 week old untreated Fisher 344 rats (n = 8). GDNF protein content was determined by ELISA. GDNF protein content of mesenteric vessels from mesenteric veins was significantly increased compared to arteries.

GDNF Protein Content in Mesenteric Vessels: LNAME Rats-12 Weeks:

GDNF protein content in NTN arteries (153.46 ± 5.8 pg GDNF/mg tissue; n = 8) and veins (1842.61 ± 483.5 pg GDNF/mg tissue; n = 8) from was significantly different than HTN arteries (415.20 ± 75.6 pg GDNF/mg tissue; n = 6) and veins (5520.19 ± 1353.2 pg GDNF/mg tissue; n = 6) from (Figure 3).

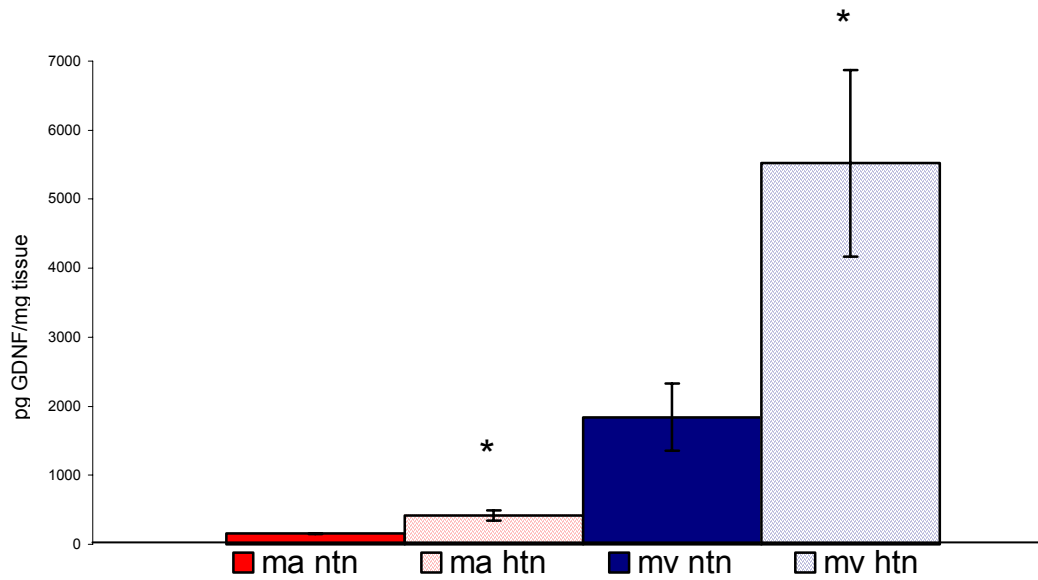


Figure 3: GDNF Protein Content in Mesenteric Arteries and Veins from 12 Wk Old LNAME Treated Fisher 344 Rats Versus Age Matched Controls. Mesenteric arteries (MA) and veins (MV) were removed from 12 week old LNAME treated Fisher 344 rats (n = 8) and age matched controls. GDNF protein content was determined by ELISA. GDNF protein content of LNAME treated vessels was significantly increased compared to vessels from untreated age-matched controls.

Isolated Cannulated Vessel Experiments-5 Weeks:

GDNF protein content in arteries pressurized to 50 mm Hg (2.48 ± 0.13 pg GDNF/mg tissue; n = 8) and veins to 4 mm Hg (2.76 ± 0.35 pg GDNF/mg tissue; n = 6) was significantly lower than arteries pressurized to 100 mm Hg (7.04 ± 0.5 pg GDNF/mg tissue; n = 8) and veins pressurized to 15 mm Hg (6.19 ± 0.35 pg GDNF/mg tissue; n = 8) from (Figure 4).

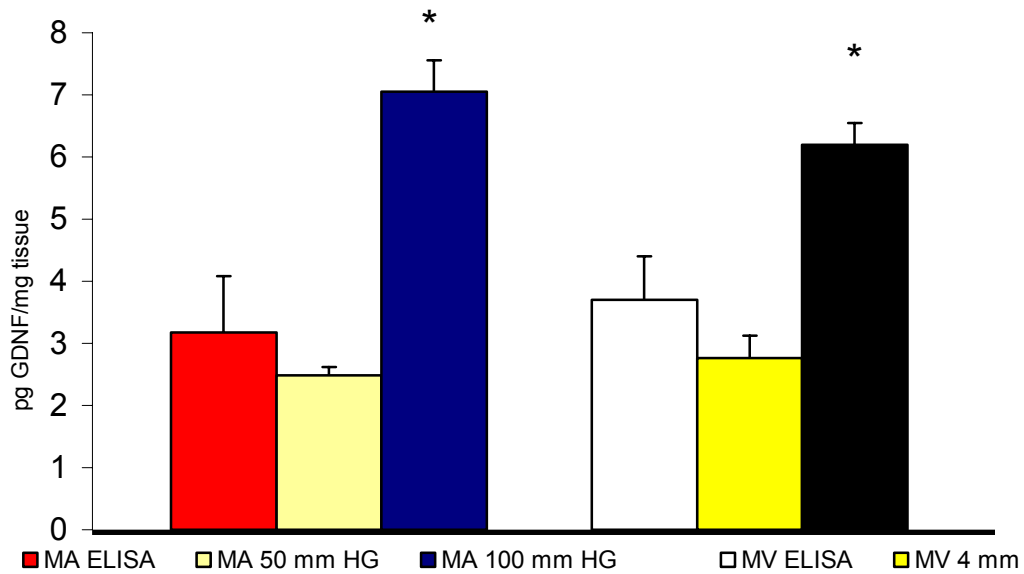


Figure 4: Isolated Cannulated Vessel Experiments in Mesenteric Arteries and Veins of 5 Wk Old Fisher 344 Rats. Mesenteric arteries and veins were hand dissected from 5-week-old untreated rats and pressurized to either physiological pressures (50 mm Hg-artery, 4 mm Hg vein) or pressures mimicking hypertension (100 mm Hg artery, 15 mm Hg vein). Following 4 hours of pressure the vessels were processed via ELISA for GDNF protein content. In both arteries and veins the GDNF protein content increased significantly with 4 hours of elevated pressure versus those held at physiological “normotensive” pressures.

Isolated Cannulated Vessel Experiments- 12 Weeks:

GDNF protein content in arteries pressurized to 50 mm Hg (2.6 ± 0.04 pg GDNF/mg tissue; n = 6) and veins to 4 mm Hg (10.86 ± 1.14 pg GDNF/mg tissue; n = 5) was significantly lower than arteries pressurized to 100 mm Hg (6.29 ± 0.97 pg GDNF/mg tissue; n = 5) and veins pressurized to 15 mm Hg (29.07 ± 7.6 pg GDNF/mg tissue; n = 6) from (Figure 5).

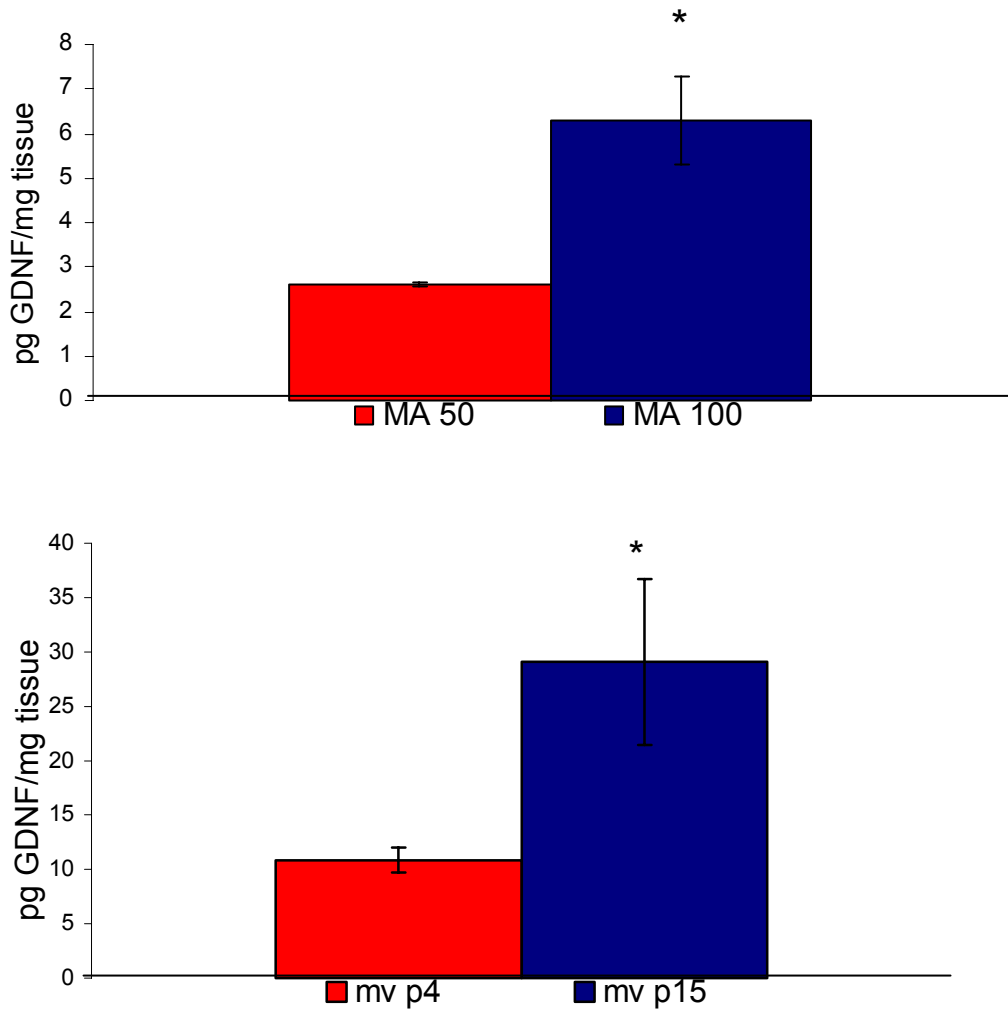


Figure 5: Isolated Cannulated Vessel Experiments in Mesenteric Arteries and Veins of 12 Wk Old Untreated Fisher 344 Rats.

Vessels of 12 wk old untreated Fisher rats were isolated, cannulated, and pressurized to either normotensive or hypertensive pressures and held for 4 hours. Following 4 hours of pressure the vessels were processed for GDNF protein content via ELISA. In both arteries and veins the GDNF protein content increased significantly with 4 hours of elevated pressure versus those held at normotensive pressures.

Immunohistochemistry:

GDNF Localization in Vessels of SS/Jr-High Salt and Low Salt Rats:

Immunohistochemical studies were done to test which nerves contained GDNF protein. Sympathetic innervation was tested by using antibodies against tyrosine hydroxylase (TH) and sensory innervation was tested by using antibodies against Calcitonin Gene Related Peptide (CGRP). The vessels from LS, HS and NTN and HTN animals all showed fibers staining positive for GDNF, CGRP and TH. In the hypertensive vessels, GDNF appeared to be contained within the fibers staining positive for TH. In the normotensive vessels, GDNF appeared to be contained within fibers staining positive for CGRP.

Vessels from SS/Jr Low Salt Animals:

In SS/JR rats LS arteries and veins GDNF and TH, were present in both arteries and veins, but did not appear to be contained within the same fibers. GDNF and CGRP were present in both arteries and veins, and appeared to be contained within the same fibers.

Vessels from SS/Jr High Salt Animals:

Vessels from HS animals showed fibers staining positive for GDNF, CGRP and TH. In contrast to the LS arteries and veins, GDNF and TH, although still present in both arteries and veins, now appeared to be contained within the same fibers. Also in contrast to the localization seen in LS arteries and veins, GDNF and CGRP, although still present in both vessels, now appeared to be contained in different fibers.

Nerve Fiber Density-SS/Jr Rats:

Nerve fiber density was also assessed in the mesenteric arteries and veins. In the LS mesenteric arteries, the density of GDNF positive arteries (162.8 ± 5.9 fibers/194 μm^2) and TH positive (92 ± 5.5 fibers/194 μm^2) fibers increased significantly between LS and HS GDNF positive arteries (244.8 ± 10.1 fibers/194

μm^2) and TH positive (317.3 ± 6.9 fibers/ $194 \mu\text{m}^2$) animals. Conversely, the fibers staining positive in mesenteric arteries for CGRP significantly decreased from LS (255.6 ± 12.8 fibers/ $194 \mu\text{m}^2$) to HS (160 ± 32 fibers/ $194 \mu\text{m}^2$). In the LS mesenteric veins, the density of GDNF positive fibers (133.5 ± 3.6 fibers/ $194 \mu\text{m}^2$) and TH positive fibers (67.3 ± 1.2 fibers/ $194 \mu\text{m}^2$) increased significantly between LS and HS; GDNF(222.1 ± 8.3 fibers/ $194 \mu\text{m}^2$) and TH positive (268 ± 9.8 fibers/ $194 \mu\text{m}^2$) fibers. There was no change in the density of fibers staining positive for CGRP in LS (184.6 ± 13.5 fibers/ $194 \mu\text{m}^2$) and HS (159.3 ± 15.8 fibers/ $194 \mu\text{m}^2$) animals (Figure 6).

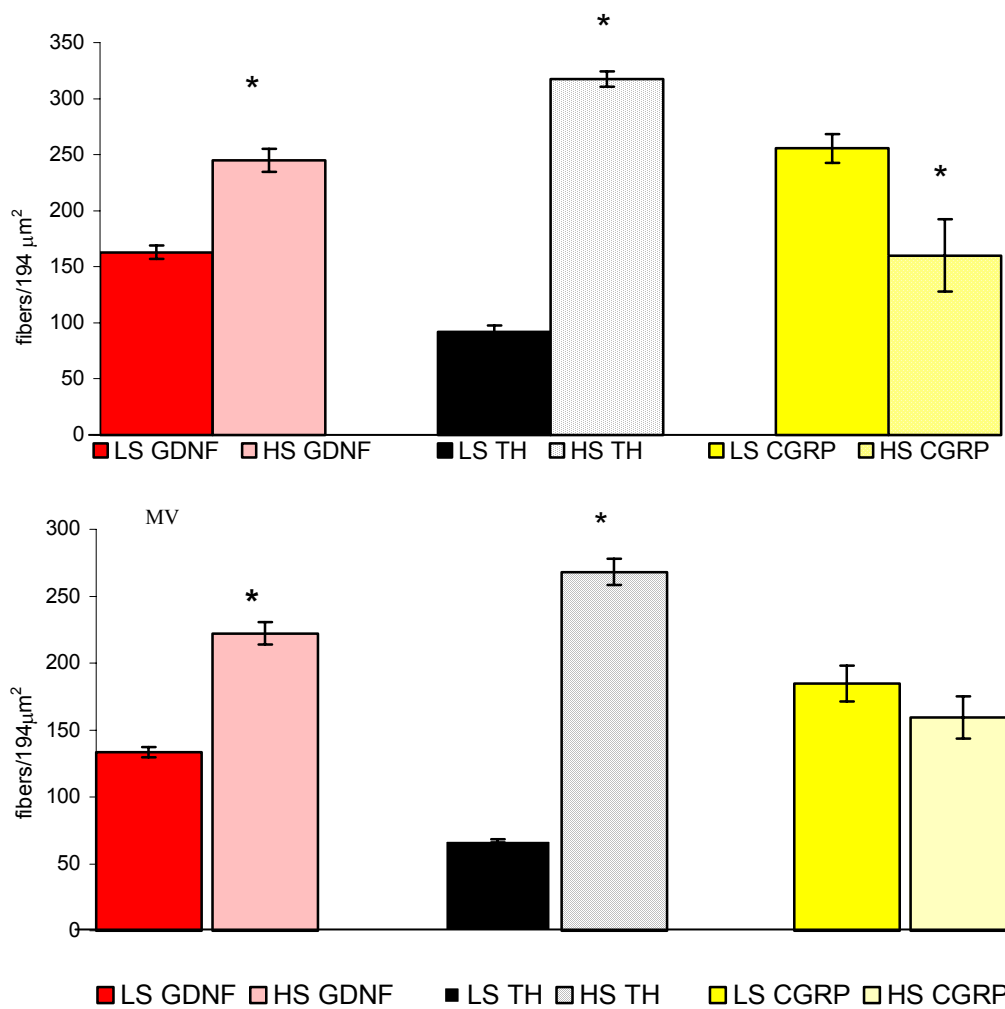


Figure 6: Immunohistochemical Staining of GDNF, TH, and CGRP Positive Fibers in Mesenteric Arteries and Veins in SS/JR Rats. Mesenteric arteries and veins were removed from 5-week-old normotensive (LS) and hypertensive rats (HS). The figure displays mesenteric arteries (top panels) and mesenteric veins (bottom panels) were reacted with antibodies against TH, a marker for sympathetic neurons, CGRP, a sensory neurotransmitter, and GDNF and which were then assessed for neurite density. The density of GDNF-, TH-, and CGRP-positive fibers was counted by placing a grid over images ($194 \mu\text{m}^2/\text{image}$) of vessels, and counting the number times that nerve fibers crossed the grid. In vessels from LS rats, both in the artery and vein the GDNF and TH neurite density increases with the development of hypertension. Conversely the CGRP content decreases over the same time.

Histology was also performed using antibodies against the receptor for GDNF, GFR α 1. In the LS (arteries-184.6 \pm 13.5 fibers/194 μm^2 as well as HS animals, the fiber crosses/194 μm^2 of GFR α 1 paralleled the fiber crossings associated with GDNF. In other words as the fiber crossings increased or decreased for GDNF, the fibers crossings for GFR α 1 increased or decreased as well.

Nerve Fiber Density-Fisher 344 – 5 Week Old-Normotensive Rats:

Nerve fiber density was assessed in the mesenteric arteries and veins of 5 wk old animals. In the mesenteric arteries, the density of GDNF positive fibers was 132.25 \pm 6.3 fibers/194 μm^2 , TH positive fibers was 82.75 \pm 5.6 fibers/194 μm^2 and CGRP positive fibers was 169.5 \pm 5.8. In the mesenteric veins, the density of GDNF positive fibers was 159.8 \pm 5.6 fibers/194 μm^2 , TH positive fibers was 107.75 \pm 4.8 fibers/194 μm^2 and CGRP positive fibers was 193.5 \pm 6.7 (Figure 7). Histology was also performed using antibodies against the receptor for GDNF, GFR α 1. The fiber crossings for GFR α paralleled the fiber crossings for GDNF in both arteries and veins.

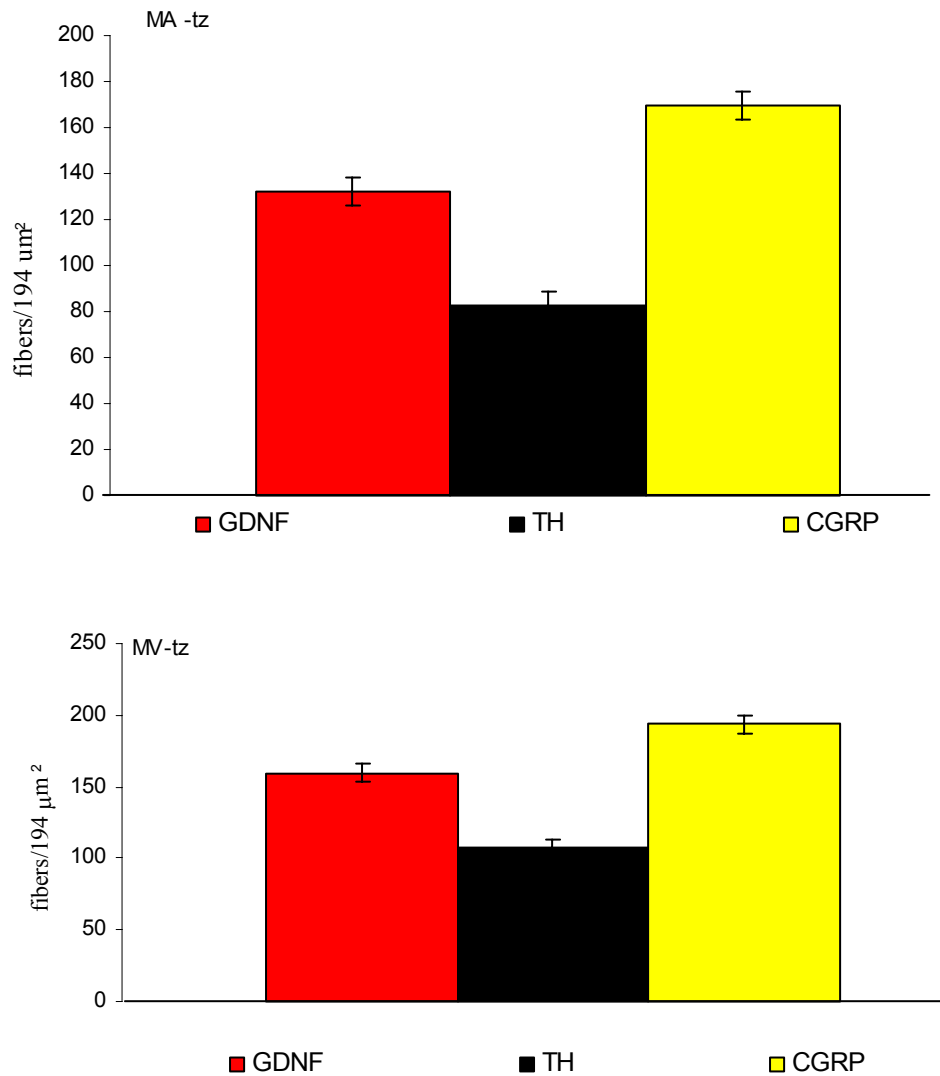


Figure 7: Immunohistochemical Staining of GDNF, TH, and CGRP Positive Fibers in Mesenteric Arteries and Veins in 5 Wk Old Fisher 344 Rats. Mesenteric arteries and veins were removed from 5-week-old normotensive Fisher 344 rats. The figure displays mesenteric arteries (top panels) and mesenteric veins (bottom panels) were reacted with antibodies against TH, a marker for sympathetic neurons, CGRP, a sensory neurotransmitter, and GDNF and which were then assessed for neurite density. The density of GDNF-, TH-, and CGRP-positive fibers was counted by placing a grid over images (fibers/194 μm^2 /image) of vessels, and counting the number times that nerve fibers crossed the grid. In vessels of normotensive rats, both in the artery and vein the density of CGRP fibers was significantly greater than TH.

Nerve Fiber Density-Fisher 344-Normotensive versus Hypertensive Rats:

Nerve fiber density was also assessed in the mesenteric arteries and veins. In the normotensive mesenteric arteries, the density of GDNF positive fibers (206.8 ± 9.4 fibers/ $194 \mu\text{m}^2$) and TH positive (136.3 ± 10.7 fibers/ $194 \mu\text{m}^2$) fibers increased significantly between normotensive and hypertensive GDNF positive arteries (327.1 ± 6.5 fibers/ $194 \mu\text{m}^2$) and TH positive (351.6 ± 2.6 fibers/ $194 \mu\text{m}^2$) animals. Conversely, the fibers staining positive for CGRP significantly decreased from normotensive arteries (386.3 ± 14.5 fibers/ $194 \mu\text{m}^2$) to hypertensive (241.6 ± 14.4 fibers/ $194 \mu\text{m}^2$). In the normotensive mesenteric veins, the density of GDNF positive fibers (195.5 ± 9.1 fibers/ $194 \mu\text{m}^2$) and TH positive (148.0 ± 6.6 fibers/ $194 \mu\text{m}^2$) increased significantly between normotensive and hypertensive animals GDNF (355.0 ± 9.8 fibers/ $194 \mu\text{m}^2$) and TH positive (358.6 ± 14.7 fibers/ $194 \mu\text{m}^2$) animals. Conversely, the fibers staining positive for CGRP decreased between normotensive (374.0 ± 3.1 fibers/ $194 \mu\text{m}^2$) and hypertensive (212.3 ± 3 fibers/ $194 \mu\text{m}^2$) animals (Figure 8).

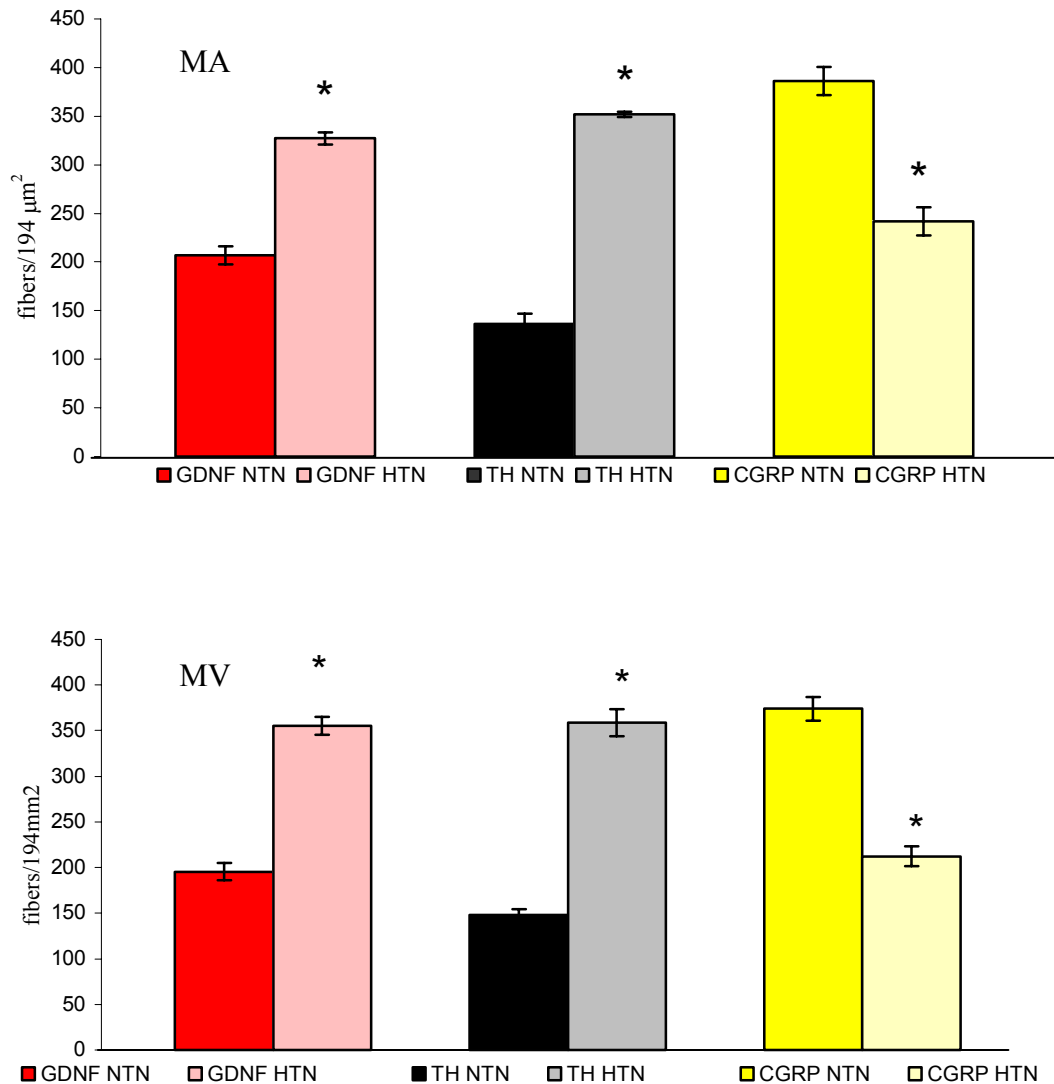


Figure 8: Immunohistochemical Staining of GDNF, TH, and CGRP Positive Fibers in 12 Wk Old LNAME Treated Mesenteric Arteries and Veins of Fisher 344 Rats. Mesenteric arteries and veins were removed from 12-wk-old LNAME treated and age-matched controls. The figure displays mesenteric arteries (top panels) and mesenteric veins (bottom panels) were reacted with antibodies against TH, a marker for sympathetic neurons, CGRP, a sensory neurotransmitter, and GDNF and which were then assessed for neurite density. The density of GDNF-, TH-, and CGRP-positive fibers was counted by placing a grid over images ($194 \mu\text{m}^2/\text{image}$) of vessels, and counting the number times that nerve fibers crossed the grid. In vessels from LNAME treated rats, both in the artery and vein the GDNF and TH neurite density increases with the development of hypertension. Conversely the CGRP content decreases over the same time.

Histology was also performed using antibodies against the receptor for GDNF, GFR α 1. In the normotensive (arteries 229.3 ± 6.1 fibers/ $194 \mu\text{m}^2$ and veins 220.0 ± 4.0 fibers/ $194 \mu\text{m}^2$) as well as hypertensive animals (arteries 400.6 ± 13.3 fibers/ $194 \mu\text{m}^2$ and veins 280.6 ± 5.7 fibers/ $194 \mu\text{m}^2$) the fiber crosses of GFR α 1 paralleled the fiber crossings associated with GDNF. In other words as the fiber crossings increased or decreased for GDNF, the fibers crossings for GFR α 1 increased or decreased as well.

Discussion:

In the current study, we have demonstrated novel findings concerning GDNF expression in two models of hypertension in the mesenteric vascular bed. The GDNF protein content of mesenteric vessels increases with increased blood pressure in both models of hypertension. In addition, when nerve fiber density was analyzed, density of TH positive fibers in vessels from hypertensive rats was significantly higher than that in vessels from normotensive rats, while CGRP positive fibers decreased. Taken together, these data suggest that as we become hypertensive, innervation patterns may shift from a balanced sensory/sympathetic innervation pattern seen in normotensive animals to a predominantly sympathetic innervation pattern as seen in hypertensive animals. These changes in protein content and innervation patterns may be a crucial step in the initiation or maintenance of the hypertensive state seen in these two models of hypertension.

It has been well established that, for NGF, both protein and mRNA expression in SHR increases with hypertension (2,24-28). This change in NGF protein expression has been linked to increases in sympathetic innervation (3,29). Increases in sympathetic innervation may be linked to overall increases in peripheral resistance and therefore blood pressure. In the current study, we have shown that GDNF protein content in the mesenteric artery and vein increases with development of high blood pressure.

In younger rats the density of innervation of both sensory (CGRP) and sympathetic (TH) markers was balanced. In both models, as the animals developed hypertension, the density of sympathetic innervation, as assayed by TH staining, increased similar to

what is seen in SHR. In addition, GDNF density and content increases with hypertension. This increase has characteristics in common with infantile hypertrophic pyloric stenosis in which there is hypertrophy and hyperplasia of the smooth muscle cells. In our study we have shown that GDNF is colocalized to smooth muscle cells (VSMC). Therefore, as the VSMC change with the development of hypertension there may be an increase in the production of GDNF. Increases in trophic factor production may lead to increased neural density, which is a hallmark of hypertension.

Results of studies on spontaneously hypertensive rats provide good evidence that alterations in vascular NGF expression can lead to changes in sympathetic (30-32) and sensory nervous system function (33). A comparable change may be associated with changes in vascular GDNF expression. Immunohistochemical analysis revealed that GDNF was found to be colocalized with TH in nerve fibers in the hypertensive animals and with CGRP in nerve fibers in normotensive animals. Calcitonin gene-related peptide (CGRP), a peptide neurotransmitter found in sensory neurons, has been shown to be a potent vasodilator (34). CGRP can be released from sensory nerve endings following a variety of stimuli, including exposure to capsaicin, with antidromic stimulation (35) or with changes in hydrogen ion content of tissues (36). It is interesting that Supowit *et al.* (33) reported a decrease in CGRP content in sensory neurons in SHR. A decrease in the level of expression of CGRP, a vascular relaxant, would help to drive blood pressure higher in hypertension.

Results of our study illustrate a potential model for the development and/or maintenance of hypertension in which an initial increase in blood pressure causes vascular stretch. Vascular stretch has been associated with increased NGF production in cultured vascular smooth muscle cells (37). This stretch may also cause an increase in GDNF. Increased GDNF expression may increase vascular innervation. Increases in vascular innervation have been associated with the development of hypertension (2,38-40). Increased sympathetic innervation may lead to increased vasoconstriction increased peripheral resistance and an overall increase in blood pressure.

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INTRODUCTION TO CHAPTER V

In addition to changes seen in the vasculature, we chose to study changes that may be observed during exercise in the heart. Changes in NGF and GDNF were analyzed. NGF and GDNF are neurotrophic factors produced by cardiac muscle. Both have potent effects on autonomic and sensory neurons. Altered expression of neurotrophic factors can have effects on nerve integrity, innervation pattern, and nervous system function. The goal of the current study was to determine whether the level of expression of NGF and GDNF protein in cardiac muscle is altered by physical activity. We hypothesized that the level of expression of trophic factors in cardiac muscle would be regulated by physical activity, such that increased activity would lead to increased neurotrophic factor production. Neurotrophic factor protein content of cardiac muscle from Fisher 344 rats was measured following voluntary running in a running wheel. Levels of expression of NGF and GDNF protein in cardiac muscle were measured via enzyme-linked immunosorbant assay. The results show that voluntary exercise increased GDNF expression in left and right atria and left and right ventricle, while only increasing NGF content in right atria. This exercise dependent regulation of neurotrophic factor expression in the heart may play a role in altered nerve structure and function observed with exercise training.

CHAPTER V

NERVE GROWTH FACTOR AND GLIAL CELL LINE DERIVED NEUROTROPHIC FACTOR PROTEIN LEVELS ARE REGULATED BY EXERCISE IN ADULT RAT HEART

Abstract:

Nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) are neurotrophic factors produced by cardiac muscle. Both have potent effects on autonomic and sensory neurons. Altered expression of neurotrophic factors can have effects on nerve integrity, innervation pattern, and nervous system function. The goal of the current study was to determine whether the level of expression of NGF and GDNF protein in cardiac muscle is altered by physical activity. We hypothesized that the level of expression of trophic factors in cardiac muscle would be regulated by physical activity, such that increased activity would lead to increased neurotrophic factor production. Neurotrophic factor protein content of cardiac muscle from Fisher 344 rats was measured following voluntary running on a running wheel. Levels of expression of NGF and GDNF protein in cardiac muscle were measured via enzyme-linked immunosorbant assay. The results show that voluntary exercise increased GDNF expression in left and right atria and left and right ventricle, while only increasing NGF content in right atria. This exercise dependent regulation of neurotrophic factor expression in the heart may play a role in altered nerve structure and function observed with exercise training.

Introduction:

Development of autonomic innervation of cardiac tissues in the rat has been shown to occur in the late prenatal and early postnatal stages of development. The degree of cholinergic innervation of the heart typically peaks around 1 to 2 weeks of age (1), while development of adrenergic (2) and intrinsic cardiac ganglia (3) appears complete by about 3 weeks of age. The level of innervation of the heart by both cholinergic (1) and adrenergic neurons (4) has been demonstrated to decline with age.

Most populations of neurons are dependent upon a continuous supply of neurotrophic factors for growth and maintenance of cell phenotype. Glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor for neurons of the parasympathetic nervous system (5-7), while nerve growth factor (NGF) is important for maintenance of normal structure and function of sympathetic neurons (8,9). Both NGF (10) and GDNF (11) have been shown to exert acute effects on neurotransmitter release and chronic effects on density of target tissue innervation (9,12,13). Thus, alterations in the level of expression of NGF and/or GDNF could exert both short- and long-term effects on nervous structure and function. Although a fair amount is known concerning regulation of expression of NGF and GDNF in glial cells (14-18), smooth muscle cells (19,20) and skeletal muscle (21,22), less is known concerning the regulation of neurotrophic factor expression in cardiac muscle. Since these neurotrophic factors have potent effects on a variety of neural parameters, it is important to understand the regulation of trophic factor expression in normal conditions and under conditions of altered activity.

Exercise training offers numerous benefits to cardiovascular health in normal and diseased hearts. In the healthy heart, exercise training leads to an increase in cardiac output, athletic hypertrophy of the left ventricle, and resting bradycardia (23,24). It is becoming evident that a rehabilitation program, including exercise training, is valuable in reducing risk of further complications following acute myocardial infarction (AMI) (25,26) and coronary artery bypass surgery (24).

The goal of the current study was to determine whether the level of expression of NGF and GDNF in cardiac muscle is altered by physical activity. For these studies,

levels of expression of neurotrophic factor proteins were measured in rat cardiac muscle following voluntary running in a running wheel. We hypothesized that the level of expression of trophic factors in cardiac muscle would be regulated in an activity dependent manner, such that increased activity would lead to increased neurotrophic factor production. We propose that the altered expression of neurotrophic factors in cardiac tissues may play a role in mediating the positive effects of exercise training on nervous system structure and function.

Materials and Methods:

Subjects:

All animal experiments were performed in accordance with the "Guide for the Care and Usage of Laboratory Animals" (National Research Council) and were approved by the Institutional Animal Care and Use Committee at Western Michigan University. Animals in the control and exercise groups were housed individually with access to food and water *ad libitum*. Animals were kept on a 12:12 hour light-dark cycle in a room where the temperature was regulated (22-24°C). All animals were monitored daily and body weights were measured weekly to ensure positive weight gain. Animals were sacrificed by CO₂-asphyxiation followed by thoracotomy.

Exercise Protocol:

Twenty four male Fisher 344 rats (Charles River, Portage MI) were used. Eight sedentary control rats (8 weeks of age) were sacrificed at the start of the study (8 wk Sed), 8 rats were housed individually in cages with free access to running wheels (22 wk VT) and 8 age-matched sedentary control rats were housed individually in cages without access to running wheels (22 wk Sed). Distance run was measured using a bicycle odometer attached to the running wheels. Following 14 weeks of running, exercised and age-matched sedentary control rats were sacrificed and tissues removed for analysis of neurotrophic factor protein levels.

Blood Pressure Measurement:

Systolic blood pressure was measured using tail-cuff plethysmography (IITC. Inc., Woodland Hills, CA). Blood pressure information from the amplifier was recorded on a chart recorder (Gould Instrument Systems Inc., Dayton, OH). Animals were restrained in tubes in a heated chamber (29°C) and given 30 minutes to acclimate prior to blood pressure measurement.

Tissue Processing:

Hearts were removed, rinsed to remove blood and separated into left and right atria and left and right ventricle. Tissue sections were flash frozen by contact with dry ice and stored at -80°C. Frozen muscle samples were dipped into liquid nitrogen and then pulverized on a metal block chilled on dry ice. The pulverized muscle was suspended in 0.1 M 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), 0.2 mM Benzamidine, 0.01 mM Benzethonium Chloride, and 0.2 mM ethylenediaminetetra-acetic acid (all from Sigma, St. Louis, MO). The suspension was chilled on wet ice while being homogenized for 30 seconds using a variable speed Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). Homogenate was centrifuged at 14,000 x g and the supernatant was analyzed using an enzyme-linked immunosorbant assay (ELISA) specific for GDNF or NGF.

Enzyme-Linked Immunosorbant Assay (ELISA) for GDNF:

GDNF content of cardiac muscle was measured by ELISA as previously described (22), with minor modifications. Briefly, 96-well plates (NUNC-ImmunoTM, Rochester, NY) were bound with monoclonal antibody against GDNF (R&D Systems, Minneapolis, MN). Remaining sites on plates were blocked with BSA (1%). Plates were washed and muscle supernatant or GDNF standard (R&D Systems, Minneapolis, MN) was added to each well. Following incubation of the sample or standard, plates were washed and bound with an anti-GDNF antibody conjugated to

biotin (R&D Systems, Minneapolis, MN). The wells were washed 3 times and beta-galactosidase conjugated to streptavidin (Molecular Probes, Eugene, OR) was added. The wells were washed and chlorophenol red- β -D-galactopyranoside (CPRG; Roche Molecular Biochemicals, Indianapolis, IN) was added and allowed to incubate for 30 minutes to 12 hours (at room temperature). Absorbance was measured (575 nm) on a microplate scanning spectrophotometer. For each assay, a standard curve was calculated from the known GDNF standard concentrations. GDNF content of tissues were reported as pg of GDNF per mg of tissue wet weight.

Enzyme-Linked Immunosorbant Assay (ELISA) for NGF:

The NGF protein content of cardiac muscle was measured by ELISA as previously described (27). Briefly, 96-well plates (NUNC-ImmunoTM, Rochester, NY) were coated with anti-NGF monoclonal antibody (Chemicon, Temecula, CA). Remaining sites on the plate were blocked BSA (1%). Plates were washed and 50 μ l/well of sample or NGF standard were added. Each sample and standard was assayed in quadruplicate. Plates were washed and bound with anti-NGF antibody conjugated to β -galactosidase (Chemicon, Temecula, CA). Plates were washed and the galactosidase substrate CPRG was added. Plates were read on a microplate spectrophotometer at a wavelength of 575 nm. NGF content of tissues were reported as pg of NGF per mg of tissue wet weight.

Statistical Analysis:

Comparisons between control and treatment were made using Analysis of Variance (ANOVA). Post hoc comparisons were made using the Student-Neuman-Keuls test. For all tests significance was set to $p \leq 0.05$. All data values are reported as the mean \pm standard error of the mean.

Results:

Gross Observations:

Body weights of voluntary runners were significantly lower (305 ± 7.7 g) than weights of age-matched sedentary control rats (325.2 ± 4.4 g). However, all animals showed positive weight gain throughout the experiments.

Distances Run:

The average distance run by the rats steadily increased over a 5 week time period, stabilizing at 250 ± 75 m/day (1.7 ± 0.5 km/week = 1.1 ± 0.3 miles per week).

Blood Pressure:

Eight-week-old sedentary rats had a systolic blood pressure of 129.6 ± 3.2 mm Hg. By 22 weeks of age systolic blood pressure in sedentary rats was significantly elevated (146.7 ± 1.9 mm Hg) compared to that in 8-week-old sedentary rats. Fourteen weeks of voluntary exercise caused a significant decrease in systolic blood pressure (124.1 ± 3.2 mm Hg) compared to that in age matched 22-week-old sedentary rats.

Changes in Nerve Growth Factor Content of Cardiac Tissues:

At the start of the exercise program, 8 sedentary control rats were sacrificed and baseline levels of NGF were measured in cardiac tissues. Figure 1A shows that in 8-week-old sedentary rats, levels of NGF protein in left and right atria (1.45 ± 0.63 pg/mg and 1.32 ± 0.30 pg/mg, respectively) were greater than that in left and right ventricle (0.3 ± 0.09 pg/mg and 0.02 ± 0.01 pg/mg, respectively). By 22 weeks of age NGF protein levels had declined significantly in left and right atria (0.03 ± 0.006 pg/mg and 0.01 ± 0.005 pg/mg, respectively) and right ventricle (0.03 ± 0.01 pg/mg), but not in the left ventricle (0.05 ± 0.005 pg/mg) (Figure 1A). Voluntary exercise, in the form of running on a running wheel, had little effect on the level of expression of NGF protein in left atria (0.06 ± 0.02 pg/mg) and left and right ventricle (0.08 ± 0.01

pg/mg and 0.04 ± 0.004 pg/mg, respectively) but lead to a significant increase in the right atria (0.05 ± 0.008 pg/mg (Figure 1B).

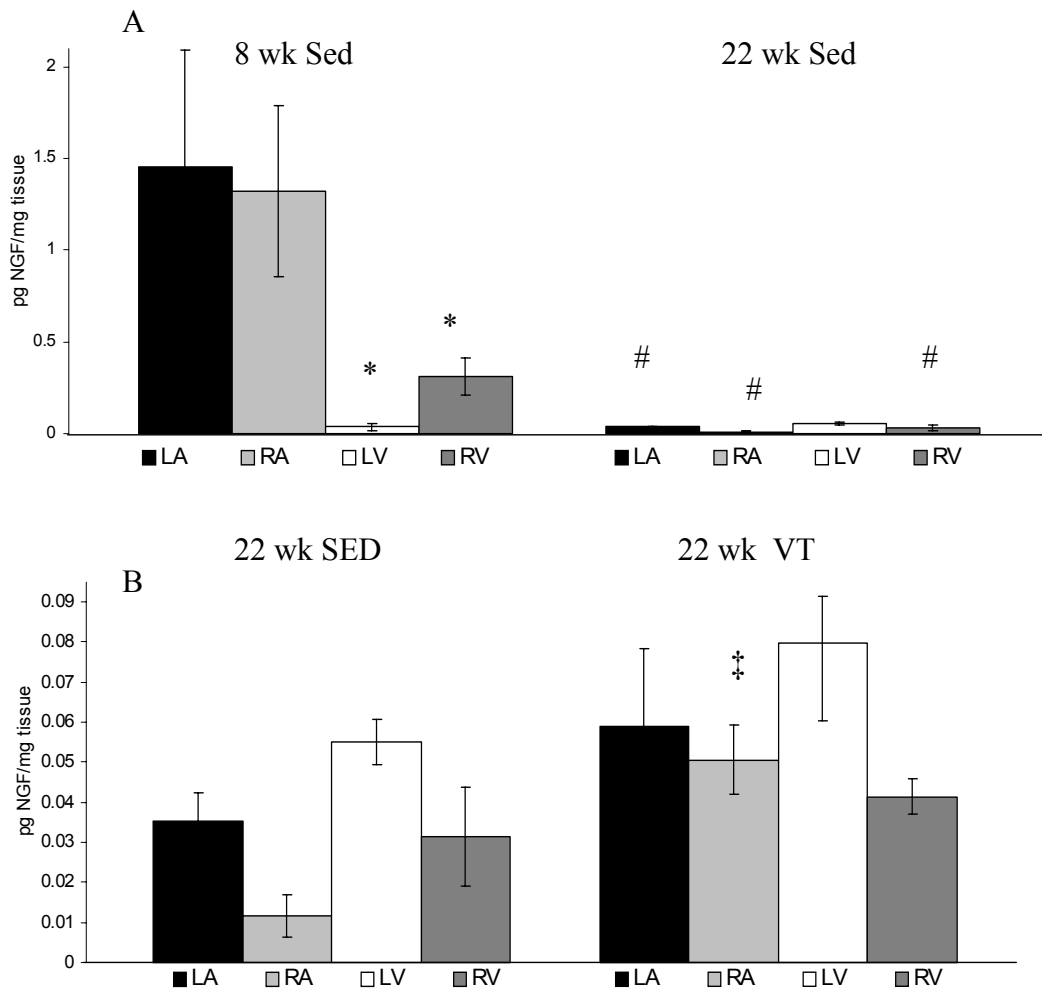


Figure 1. Nerve Growth Factor Protein Content in Cardiac Muscle from 8- and 22 Wk Old Rats and from 22 Wk Old Rats Following 14 Wks of Voluntary Running. Hearts were removed from 8- (8 wk Sed) and 22-week-old sedentary rats (22 wk Sed) and 22-week-old rats following 14 weeks of voluntary running in a running wheel (22 wk VT). NGF protein content of atria and ventricle was measured via ELISA.

Figure 1A. NGF protein content in 8-week-old sedentary animals is significantly lower in the left (LV, *) and right (RV, *) ventricle versus the left (LA) and right atria (RA). NGF protein content in 22-week-old sedentary animals is significantly decreased in left (#) and right (#) atria and right ventricle (#) versus the levels in tissues from 8 wk old sedentary animals.

Figure 1B. Voluntary exercise (14 weeks of running in a running wheel) led to significant increase in NGF protein in right atria (‡) but not in left atria or ventricles. All significance set at $p \leq 0.05$. Values are means \pm SEM.

Changes in Glial Cell Line Derived Neurotrophic Factor Content of Cardiac Tissues:

Figure 2A displays levels of GDNF protein in left and right atria (0.44 ± 0.18 pg/mg and 1.17 ± 0.2 pg/mg, respectively) and left and right ventricle (0.83 ± 0.08 pg/mg and 1.14 ± 0.09 pg/mg, respectively) from the 8-week-old sedentary rats. From this figure it can be seen that the left atria and left ventricle had significantly less GDNF than right atria and right ventricle. From figure 2A it can be seen that the concentration of GDNF protein increased in left and right atria (2.01 ± 0.48 pg/mg and 2.14 ± 0.08 pg/mg, respectively) between 8 and 22 weeks of age. The concentration of GDNF protein in right ventricle (0.66 ± 0.12 pg/mg) decreased over this same time (Figure 2A). Voluntary exercise, in the form of running in a running wheel, led to an increase in the level of expression of GDNF protein in left and right atria (12.1 ± 1.1 pg/mg and 9.15 ± 1.69 pg/mg, respectively) and left and right ventricle (2.29 ± 0.37 pg/mg and 1.46 ± 0.07 pg/mg, respectively) (Figure 2B).

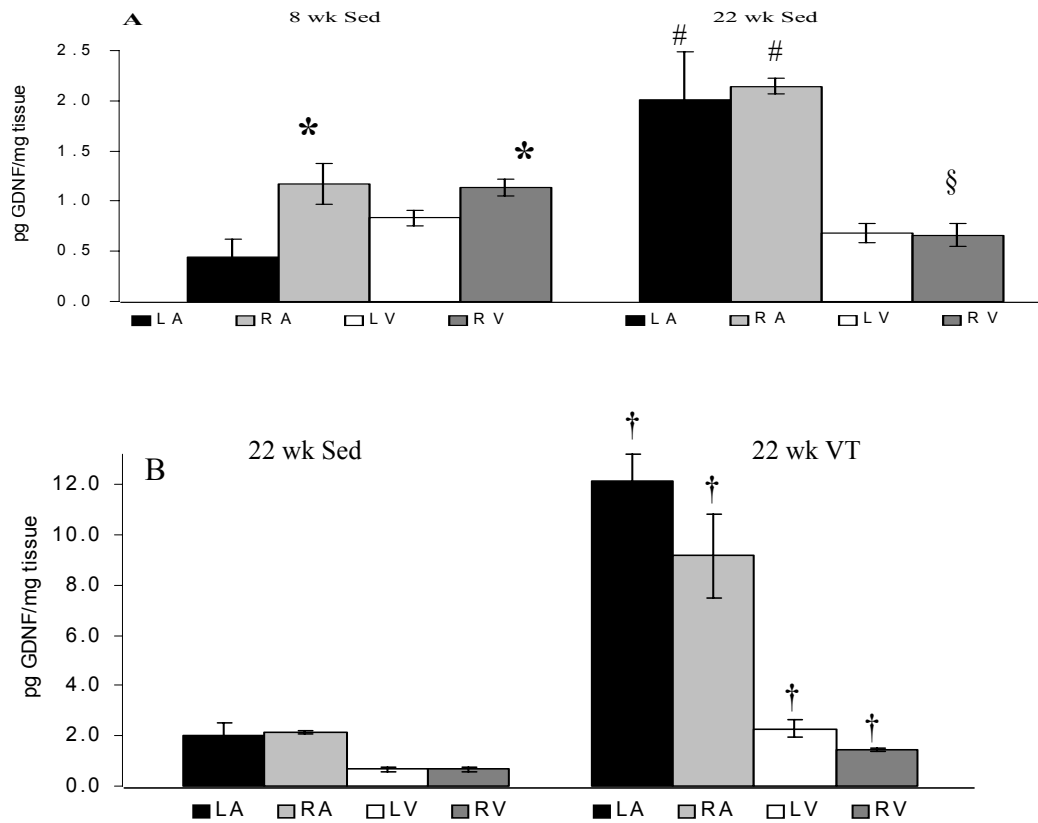


Figure 2. Glial Cell Line Derived Neurotrophic Factor Protein Content in Cardiac Muscle from 8- and 22 Wk Old Rats and from 22 Wk Old Rats Following 14 Wks of Voluntary Running.

Hearts were removed from 8- (8 wk Sed) and 22-week-old sedentary rats (22 wk Sed) and 22-week-old rats following 14 weeks of voluntary running in a running wheel (22 wk VT). GDNF protein content of atria and ventricle was measured via ELISA. **Figure 2A.** In 8-week-old sedentary rats, GDNF protein content in the right atria (RA, *) and right ventricle (RV, *) is significantly greater than that of left atria (LA) and left ventricle (LV). In 22-week-old sedentary rats, GDNF protein content in left and right atria (#) is significantly elevated compared to levels in left and right atria from 8-week-old sedentary animals, while GDNF protein content in right ventricle (§) declined significantly between 8 and 22 weeks of age. **Figure 2B.** GDNF protein content in the atria and ventricles of 22-week-old exercised animals is significantly greater (†) than that in the atria and ventricles of 22-week-old sedentary animals. All significance was set at $p \leq 0.05$. Values are means \pm SEM.

Discussion:

Cardiac tissues have been shown to express several neurotrophic factors including NGF (28,29) and GDNF (30). NGF plays a vital role in the maintenance and survival of sympathetic and sensory neurons (8,31), while GDNF plays a role in survival and function of sympathetic, parasympathetic and sensory neurons (5-7,32). Results of the current study show that exercise training, in the form of voluntary running in a running wheel, resulted in a significant decrease in blood pressure and an increase in GDNF protein expression in all chambers of the heart and an increase in NGF protein expression in the right atria. These findings suggest that levels of expression of NGF and GDNF protein in cardiac muscle are regulated by physical activity.

Studies examining NGF expression in cardiac muscle provide conflicting results concerning changes in the level of expression with age. Several studies have shown levels of expression of NGF mRNA (33) and protein (34) to decline in cardiac muscle to low stable levels of expression by 3 weeks of age, at which time the degree of sympathetic innervation also reaches adult levels (2). However, Stuerenburg and Kunze found that NGF protein levels in rat heart increase up to 15 months of age and decrease thereafter (35). Our finding that NGF protein levels in left and right atria and right ventricle decline dramatically between 8 and 22 weeks of age fits somewhere between these previous findings and may suggest a continued developmental role for NGF in atrial tissues until at least 8 weeks of age.

Most studies examining GDNF expression in cardiac tissues have also demonstrated age-related declines in neurotrophic factor expression (36-38). We also demonstrated an age-related decline in GDNF content in ventricle, but not in atria, where GDNF content increased between 8 and 22 weeks of age. One potential explanation for the discrepancy between our findings and previous reports is that many of the previous studies examined GDNF content in whole heart and may thus have missed changes in atria. Due to the very small mass of the atria, any increase in neurotrophic factor content could have been masked by declines in ventricular

tissues. These findings may also suggest a continued role for GDNF in atrial tissues through 22 weeks of age.

Our voluntary running protocol caused few changes in NGF levels in atria and ventricle, with right atria showing the only significant increase. Conversely, voluntary running led to significant increases in GDNF content in left and right atria and left and right ventricle. It is interesting to note that the rats undergoing voluntary running also showed a significant decrease in blood pressure compared to the age-matched sedentary controls. If GDNF 's role in the heart is to support the parasympathetic nervous system (5,6), then elevated expression of GDNF with exercise may enhance parasympathetic nervous system function, which may help to lower blood pressure.

Cardiac transplant results in total denervation of the heart musculature (39,40). It is of great interest to promote or enhance reinnervation of allografts by both sympathetic and parasympathetic nervous system. It is clinically significant that sympathetic neurons can reinnervate the left ventricle (41). Both NGF (42) and GDNF (36) have been shown to play a role in reinnervation of denervated cardiac muscle. If levels of expression of NGF and GDNF are increased with exercise, then it is possible that an exercise-training program could facilitate sympathetic and parasympathetic reinnervation of cardiac tissues.

There is often a disturbance of autonomic balance following acute myocardial infarction, leading to complications including sudden cardiac death and serious ventricular arrhythmias (43,44). The recovery of autonomic balance is important in preserving normal cardiac function. After acute myocardial infarction there is a shift in the balance towards an increased sympathetic drive, an obligative increase in heart rate and increased presence of arrhythmias (26). It has been shown that exercise enhances parasympathetic tone and restores the imbalance of autonomic function by increasing vagal influence, leading to a lower heart rate (26,45). Under the conditions used in these studies we saw a significant increase in GDNF protein content in all chambers of the heart, while NGF protein content was only elevated in right atria. If GDNF is acting predominately on the parasympathetic nervous system and NGF on

the sympathetic nervous system, then our exercise regimen may be expected to enhance parasympathetic activity, while having less effect on sympathetic activity, which could result in a shift in balance towards a greater parasympathetic role in control of cardiac activity.

A variety of mechanisms could underlay the effects of exercise on neurotrophic factor protein expression in cardiac muscle. It is known that an increase in mechanical load and stretch on the heart can lead to changes in gene expression (46). Exercise training results in increased stretch on the heart walls through increased venous return and cardiac output. Mechanical load regulates several pathways leading to increased transcription and/or translation. For example, there is an activation of a protein kinase C pathway that has been shown to stimulate protein synthesis in cardiac muscle cells (46) and bladder (47). There is also an increase in cAMP and cAMP dependent protein kinase activity following stretch (46). Another possible link between load and protein synthesis are stretch activated ion channels (46). Finally, there are other paracrine or autocrine mechanisms that may play a role in regulating trophic factor expression (46).

The activity dependent regulation of neurotrophic factor expression in the heart has powerful implications. The neurotrophic factors NGF and GDNF exert both short- and long-term effects on neurons and it is clear that any change in neurotrophic factor protein can have effects on nervous system structure and function. Results of the present study suggest that increased levels of physical activity lead to elevated expression of NGF and GDNF protein in cardiac tissues. In the short term, elevated expression of NGF and GDNF may play a role in modulating ongoing levels of activity in sympathetic and parasympathetic nervous system. In the long term, elevated expression of NGF and GDNF could play a role in fostering reinnervation of damaged or transplanted cardiac tissues. Further work is required to elucidate the role of altered neurotrophic factor expression in exercise-induced changes in neural architecture and nerve activity in the heart.

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CHAPTER VI

DISCUSSION OF DISSERTATION

The main goal of these studies was to determine whether the level of expression of neurotrophic factors in cardiovascular tissues changes with age, exercise and development of high blood pressure. We were able to measure GDNF and NGF protein content in both mesenteric arteries and veins, with higher levels of neurotrophic factor protein being found in the veins. In general, levels of neurotrophic factor expression in mesenteric arteries and veins decreased with age and increased with exercise. Blood pressure increased with age to hypertensive ranges in sedentary animals but decreased to normotensive ranges with exercise. In mesenteric vessels from sedentary rats, NGF and GDNF were found to colocalize to sympathetic fibers, while in vessels from young rats and mature exercised rats NGF and GDNF were found to be colocalized to sensory fibers. GDNF protein content and sympathetic nerve fiber density fibers in the mesenteric vascular bed increased with the development of hypertension. In addition, *in vitro* vessels exposed to an acute bout of elevated pressure had a significant increase in GDNF protein content. In cardiac tissue it was observed that there was an increase in GDNF protein expression in all chambers of the heart and an increase in NGF protein expression in the right atria with exercise.

GDNF is an important guidance factor in the innervation of numerous target tissues. For example, gastrointestinal smooth muscle makes GDNF (1) effects sensory and motor nerves innervating the bowel. GDNF is also a trophic factor that guides autonomic neuronal innervation to airway smooth muscle(2) and aids in the development of dorsal root sensory neurons (3). GDNF also promotes sympathetic and sensory fibers to innervate vascular tissues (4), aids in innervating skeletal muscle(5-8) and directs axonal growth in culture after spinal cord injury of propiospinal nerves and in myelination(9). Studies conducted after dorsal root injury indicated that chronic administration of GDNF resulted in sensory axons restoring connections with their target tissue, restoring sensory function (10-13). These findings indicate that GDNF is an important trophic factor which plays a role in the mapping and innervation of neurons to numerous target tissues. Despite the

importance of GDNF in the innervation of these tissues, and the importance of neural innervation in the regulation of mesenteric blood flow (14-17), no studies to date have determined if GDNF is present in mesenteric vasculature. My data indicate that GDNF is expressed in both mesenteric arteries and veins, changes with the development of hypertension and therefore, GDNF may be playing a role in changing innervation patterns seen during the development of hypertension in the rat.

It is well established that NGF is important for the survival of neurons. For instance, NGF is responsible for the survival of sympathetic neurons (18-20). NGF is also important in the survival and maintenance of sensory neurons(21-23) and guiding of sympathetic (18,24-26) and sensory neurons(22,27,28) to their targets. NGF has also been implicated in hyperinnervation of the vasculature in SHR and this hyperinnervation is thought to contribute to the development of hypertension (29-36). Since the mesentery receives about 70% of the cardiac output and is very important in blood flow regulation, I set out to determine whether NGF was in the mesenteric vasculature. My data indicate that NGF protein is present in the mesenteric arteries and veins and NGF content changes the neuronal fiber type it is expressed in with maturity and with exercise. Unlike GDNF, NGF was not contained within the VSMC but was present in the vessel, thus it is likely it was contained in the endothelial cells. NGF has been shown to be produced in mouse aortic endothelial cells and NGF's production was enhanced by proinflammatory cytokines(37-39). In endothelial cells, NGF may be serving in a regenerative or neuroinflammatory function to aid overall endothelial maintenance and function or in response to hypertension or trauma (28,37,38,40-44).

Although there have been no studies indicating that GDNF decreases with aging in the vasculature, there is precedence for other trophic factors decreasing with age as well as GDNF decreasing in other systems(45). Several studies have shown that as an organism ages NGF content decreases in dorsal root ganglion, middle cerebral arteries, basal forebrain neurons, hippocampal neurons, spinal motoneurons and aorta (20,46-50). Nerve fiber loss in cerebral blood vessels (20) and decreased

nerve regeneration due to decreases in trophic factor content have been shown with increased age (51). Several studies have shown levels of expression of NGF mRNA (52) and protein (36) to decline in cardiac muscle to low stable levels of expression by 3 weeks of age, at which time the degree of sympathetic innervation also reaches adult levels (53). I observed that GDNF and NGF protein content decreased in mesenteric vessels from mature rats. As these animals aged they became slightly hypertensive. In hypertension endothelial cells, which produce NGF, become dysfunctional. Therefore this may imply that with age the endothelial cells may lose the ability to produce NGF and there is a subsequent decrease in NGF delivered to the vasculature. Another possible reason for decreased neurotrophic protein content may be due to an increase in innervation density. The increase in density may result in increased shipment of the protein out of the vessel faster due to a greater demand. These data necessitate further research to determine the mode of action.

As an animal develops and matures there may be various trophic factors upon which the neurons may depend for survival and support. During development, the sympathetic nervous system may be more dependent on BDNF or NGF, but as these neurons mature they become more dependent on GDNF(54). Early in the development of the enteric nervous system, neurons of the neural crest only respond to GDNF, but by E14 have switched over some dependence to NT3(55). These findings imply that as the nervous system develops and matures, the dependence on certain neurotrophic factors may change with the demand, age, or state of the system. Despite the importance of trophic factor support for the nervous system, little is known concerning neurotrophic factor dependence in neurons innervating the mesenteric vasculature. My data indicate that GDNF and NGF are colocalized to nerve fibers staining positive for CGRP in mesenteric vessels from 8 week old rats; in mesenteric vessels from 22 week old sedentary rats GDNF and NGF are found primarily in nerve fibers staining positive for TH, while GDNF and NGF are once again found primarily in nerve fibers staining positive for CGRP in mesenteric vessels from 22 week old rats exercised rats. Thus there appears to be a balance

between sensory and sympathetic innervation in young, normotensive rats, with sensory neurons relying heavily on both neurotrophic factors. As the animals mature and become hypertensive the sympathetic nervous system appears to have a greater demand for neurotrophic factor and may now be using GDNF for additional trophic support (56). These data may suggest that as the animals mature, one part of the nervous system may begin to dominate and require more trophic support. The fact that the fiber dependence changed with age and exercise denotes that although the animals were hypertensive the system was still plastic enough to return to a normotensive state. This tells us that the adult nervous system is more plastic than we suspected and that although the hypertensive type connections had begun to be established with sedentary behavior, the ability to possibly reverse these connections may be attained through exercise.

There have been several studies in which arteries and veins were shown to respond differently to a variety of stimuli, including NPY, substance P, prazosin, and neural stimulation (57-61). These findings suggest that different tissues that appear to be similar in nature may have different innervation patterns. Interestingly, in all of my studies I observed higher GDNF protein content in the mesenteric veins versus the mesenteric arteries and different innervation patterns for the artery versus the vein. One possible explanation for differences in innervation patterns could be simply due to structural differences between an artery and vein. A mesenteric artery has four layers of smooth muscle cells surrounding it versus the single layer in the vein. In canine mesenteric vessels, structural differences in the arteries versus the veins include a greater number of VSMCs, more tightly packed VSMCs, and variations in the amount of contractile proteins found in each vessel(62). These differences may account for differences in vessel innervation and neurotrophic factor protein content observed in my studies.

Exercise has been linked to increases in neurotrophic factor expression and reduced in the incidence or severity of neurodegenerative disorders in the elderly(63). The ability to aid in brain self repair following injury has also been attributed to

exercise(64). Exercise has also been shown to have protective effects against neurodegenerative diseases(64). In a study conducted in the elderly it was discovered that decreases in neurotrophic factor support have been linked to dementia and depression(65). These data suggest that a beneficial effect of exercise may be to increase neurotrophic factor content, which could aid in neural repair and protect against neurodegenerative disorders. My data show an increase in GDNF and NGF protein content, following 14 weeks of voluntary exercise and a decrease in blood pressure versus the age-matched sedentary controls. These data suggest that there may be a decrease in neurotrophic factor support as the organism ages and that exercise may provide a way to bolster trophic factor support for the organism's nervous system.

It has been well documented that increased trophic factor content leads to hyperinnervation of the blood vessels(66,67). For instance, overexpression of GDNF leads to hyperinnervation of neuromuscular junctions in neonatal mice(7,68). In studies conducted with normotensive rats, infusion of NGF causes hyperinnervation of the vasculature (66,67). In studies involving SHR, NGF is thought to be responsible for increases in sympathetic innervation leading to hyperinnervation of the vasculature and development of hypertension(29,30,32-35,69-72). Overall these findings illustrate that overexpression of neurotrophic factors is important in innervation of several tissues. In light of these findings, an *in vivo* approach was taken to try to elucidate if increased GDNF protein content of the mesenteric vessels is associated with the development or maintenance of hypertension. In both the SS/Jr and the LNAME treated rats, GDNF protein content increased with the development of hypertension. These data imply that the increase in GDNF protein may be a secondary effect of hypertension and not the cause of the hypertension, although a time course study would be necessary to elucidate the order in which these events occur. As with NGF, GDNF may be acting to promote hyperinnervation of the vasculature, and therefore overexpression in the vasculature could be a mechanism through which development of hypertension is enhanced.

It has been noted that even a brief elevation in blood pressure in humans can predispose them to the development of hypertension(73,74). Thus, with the *in vitro* experiments I wanted to examine whether a brief elevation in blood pressure leads to an increase in vascular neurotrophic factor content. Results of these experiments showed that GDNF content increased in the vessels which were held at pressures mimicking a hypertensive state. Moreover, changes in GDNF protein content were observed after only four hours of elevated pressure. These data suggest that the development of hypertension may be initiated by simple physical stretch of the vasculature exposed to higher pressures. My findings suggest that short term changes in pressure may be sufficient to initiate changes in neurotrophic factor expression. If these changes in neurotrophic factor expression cause changes in nervous system structure or function, then a short term change in pressure may initiate the series of events that lead to changes in the nervous system, which are possibly long-lived.

Decreases in neurotrophic factors such as NGF and BDNF may be implicated in the initiation of cardiovascular disease(75). Most studies examining GDNF expression in cardiac tissues have demonstrated age-related declines in neurotrophic factor expression (14,76,77). In the final study I chose to examine the affects of exercise on GDNF and NGF protein content of the four chambers of the heart. My data showed an age related decline in NGF and GDNF protein in cardiac tissue. Declines in GDNF were observed in the ventricles, but not atrias where GDNF content increased between 8 and 22 weeks of age. One potential explanation for the discrepancy between my findings and previous reports is that many of the previous studies examined GDNF content in whole heart and thus may have missed changes in atria. Due to the very small mass of the atria, any increase in neurotrophic factor content could have been masked by declines in ventricular tissues.

GDNF plays a role in supporting the parasympathetic nervous system in the heart(78,79). The parasympathetic nervous system is involved in lowering heart rate and blood pressure(80,81) Thus if GDNF 's role in the heart is to support the parasympathetic nervous system, then elevated expression of GDNF with exercise

may enhance parasympathetic nervous system innervation of the heart, which may help to lower blood pressure. My findings showed that following 14 weeks of voluntary exercise there was a significant increase in GDNF content in left and right atria and left and right ventricle. Exercised animals also displayed a significant decrease in blood pressure. These data imply that through exercise the increase in GDNF may aid in decreasing overall blood pressure which may help to prevent cardiovascular disease.

Results of my studies have several implications. Although most studies done on hypertension focus on arterial blood flow, venous regulation may play an important role in maintenance of systemic blood flow. Veins carry about 70% of the body's blood volume and a slight increase in sympathetic tone may cause venoconstriction leading to increased blood pressure. In the deoxycorticosterone acetate (DOCA)-salt and SHR models of hypertension, it has been shown that there is increased venous tone (82). The observed increase in GDNF expression in mesenteric veins could possibly lead to increased sympathetic innervation and increased venous tone and a worsening of hypertension.

In order to understand blood pressure regulation, additional studies must be conducted to decipher the signaling mechanism by which increases in neurotrophic factor expression occur. This may be accomplished through studies involving the use of pharmacological agents which upregulate GDNF production, such as phorbol ester which upregulates protein kinase C (PKC), or one that inhibits GDNF's effects such as calphostin C and bisindolymaleide. These agents could be used determine the relationship PKC plays in GDNF upregulation. Also studies using antibodies to GDNF could be done to determine if increased protein production could be inhibited and therefore the development or maintenance of hypertension could be reversed or blocked.

In addition to gaining knowledge into some of the basic differences between how arteries and veins contribute to regulating blood flow, understanding how neuronal control affects resistance and capacitance vessels exemplifies one of the

major controlling factors for regulation of blood pressure and overall cardiovascular homeostasis. Results of these studies may provide some answers into which mechanisms may be important in hypertension development, maturity, and the beneficial effects of exercise. Employing the use of exercise, one may be able to reverse or condition the cardiovascular system with increased neurotrophic support which will in turn offer neuroprotection from ailments that attack the cardiovascular system.

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