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Effects of Age and Exercise on Density of Sympathetic Innervation and Localization with Nerve Growth Factor and Glial Cell Line-Derived Neurotrophic Factor in Vascular Tissue

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Effects of Age and Exercise on Density of Sympathetic Innervation and Localization with Nerve Growth Factor and Glial Cell-Line Derived Neurotrophic Factor in Vascular Tissue

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Abstract

Hypertension is a condition that affects nearly 75 million people in the United States (Merai et al, 2017). To better understand this mostly idiopathic condition, the role of survival factors on arterial innervation must be understood. Nerve growth factor (NGF) and glial cell-line derived neurotrophic factor (GDNF) have been shown to support development and maintenance of the sympathetic nervous system. The aim of this study was to reveal how density of sympathetic innervation changes in mesenteric arteries over time and with six-months of exercise. Additionally, this study aimed to reveal the localization of GDNF and NGF alongside these changes in innervation. To accomplish this, density of sympathetic innervation was measured using ImageJ software in Sprague Dawley rats. Densities were measured in an exercised and sedentary group of one-year-old rats, an exercised and sedentary group of 18-month-old rats, and a group of four-week-old sedentary rats. The experiment revealed no significant changes in density of sympathetic innervation with exercise in both the one-year-old and 18-month-old groups. The density of sympathetic innervation was significantly less in the sedentary 18-month-old group than in the four-week-old group. There was no difference in density of innervation between the four-week-old group and the 18-month-old exercised group suggesting that exercise blocked the decrease in sympathetic innervation. There was also no difference in the density of innervation between the four-week-old group and either of the one-year-old groups. GDNF and NGF do not appear to be localized within sympathetic innervation. NGF appears to be localized around adipocytes but more research must be conducted to confirm this result and its implications.

Introduction

In the United States, cardiovascular disease kills more than 2200 people each day (Mozaffarian et al, 2016). Cardiovascular disease includes both heart attack and stroke, which are often preceded by a disease state known as hypertension. Hypertension is defined as a blood pressure at or above 140/90 mm Hg. Hypertension affects nearly 75 million people in the United States alone (Merai et al, 2017). Currently, over 90% of hypertension is characterized as primary hypertension, whose underlying causes are not attributed to any specific disease (Sherwood, 2016). Primary hypertension tends to increase gradually over time with age and lack of exercise, but root causes for this condition are not well understood. Thus, to illuminate a major precursor to cardiovascular disease, possible control mechanisms behind hypertension must be explored.

Arteries and veins are controlled both locally and by the nervous system. One branch of the nervous system, known as the autonomic nervous system, is responsible for unconscious signals influencing the movement of target tissues. The autonomic nervous system is divided into two components: the sympathetic nervous system and the parasympathetic nervous system. Sympathetic innervation tends to promote the "fight or flight" response while parasympathetic innervation tends to promote a "rest and digest" response. Most tissues are innervated by both systems. However, arteries are innervated primarily by sympathetic nerves and do not hold significant parasympathetic innervation. Nerves tend to synapse onto a target tissue and transmit signals to that target tissue using neurotransmitter. Arteries are also innervated by afferent innervation, which transmits sensory information to the brain for processing. Sympathetic innervation of vessels stimulates can stimulate constriction of arteries, leading to higher blood pressure.

To gain proper neuronal innervation, target tissues secrete survival factors that promote innervation of that target tissue. Two important survival factors for the autonomic nervous system include glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF). Upon its discovery, NGF was shown to play a crucial role in supporting the development and survival of sympathetic innervation (Levi-Montalcini and Hamburger, 1951). It is hypothesized that an increase in sympathetic innervation may lead to increased vessel constriction, and therefore increase blood pressure over time. NGF protein content has been shown to increase during pulmonary hypertension (Freund-Michel et al, 2015). However, the precise mechanisms by which NGF affects the integrity and pattern of sympathetic innervation in the vasculature are not well understood.

Glial cell line-derived neurotrophic factor (GDNF) is a potent survival factor for the autonomic nervous system, the motor nervous system, and the sensory nervous system (Rebimbas-Cohen, 2005). In GDNF knockout mice, subsets of the sympathetic nervous system developed poorly or not at all (Moore et al 1996). GDNF has been shown to be regulated by physical activity in skeletal muscle (Wehrwein et al, 2002). It is not known how exercise or age affect GDNF's effects on sympathetic innervation in the vasculature.

Along with neurotrophic factor influences, activities like exercise and age also play a role in the maintenance of healthy vasculature. As individuals age, they are more likely to develop conditions like hypertension (Kang and Bodary, 2014). Exercise has been shown to lessen this effect (Seals et al, 2008) suggesting that exercise may be a preventative therapy for conditions like hypertension.

The mechanisms by which NGF and GDNF influence the integrity of sympathetic innervation of arteries is not well understood. This study aims to reveal how density of sympathetic

innervation changes with age and exercise. Additionally, this research aims to reveal patterns of NGF and GDNF localization with sympathetic innervation of arteries. The current hypothesis for this project states that density of sympathetic innervation will increase with age since blood pressure also increases with age. Increased density of sympathetic innervation could produce a high blood pressure state, as more sympathetic fibers could stimulate vessels to constrict more, producing higher arterial blood pressure. Additionally, the hypothesis states that density of sympathetic innervation will be less in groups who have undergone a six-month period of exercise than age-matched sedentary groups. Exercise has been shown to lessen the instance of high blood pressure (Seals et al, 2008), so exercise may act in reducing density of sympathetic innervation and thus reducing chronic arterial constriction associated with high blood pressure. The hypothesis regarding localization states that NGF and GDNF will be localized within smooth muscle cells in order to maintain a healthy innervation pattern.

Materials and Methods

I. Use of Vertebrate Animals in Research

All animals used in this study were maintained according to protocol approved by Western Michigan University's Institutional Animal Care and Usage Committee (IUCAC). Before completing studies using animals, all researchers completed the Collaborative Institutional Training Initiative (CITI) for Research Involving Animals.

II. Subjects

This study consisted of three animal groups of three different ages. First, six four-week-old Sprague Dawley rats were obtained and euthanized. Then, twelve six-month-old Sprague Dawley rats were randomly placed in exercised (n=6) and sedentary groups (n=6). Exercised rats

were placed into a cage alone with voluntary access to a running wheel. Distances ran by each rat were monitored throughout using software. Sedentary rats were placed in cages without access to a running wheel. Both groups were maintained for six months with access to food and water *ad libitum (*Rebimbas-Cohen, 2005). Both groups were euthanized at one year of age. Tissues were collected immediately after animals were sacrificed. Then, twelve sedentary one-year-old rats were divided into exercised (n=6) and sedentary (n=6) groups. Rats in the exercised group were placed in cages with voluntary access to running wheels. Distances ran by the exercised group were monitored using software. Sedentary rats were placed in cages without access to running wheels. Both groups were maintained for six months with access to food and water *ad libitum (*Rebimbas-Cohen, 2005). Both groups were euthanized at 18 months of age. Tissues were collected immediately after animals were sacrificed.

III. Tissue Collection

Animals were asphyxiated using a CO_2 chamber. A thoracotomy was performed, and the small intestines removed. Intestines were rinsed in phosphate buffered saline (PBS) pH 7.2. Intestines were pinned out to reveal mesenteric vein and artery pairs. It should be noted that there was no discrimination between area of the small intestine. In other words, tissues were not collected specifically from the ileum, duodenum, or jejunum but rather collected generally throughout the small intestine. Fat and connective tissue were trimmed away from a mesenteric vein and artery pair using a light microscope. Two pairs of similar size and pattern were removed from each animal and fixed in 4% paraformaldehyde for 15 minutes. Tissues were rinsed in PBS and flash frozen in -80°C.

IV. Immunohistochemistry of Whole-Mount Vessels

Tissues were placed in a blocking solution consisting of 5% sucrose and 1% Bovine Serum Albumin (BSA) for one hour. One tissue from each animal was stained for sympathetic innervation and GDNF, and the other tissue from each animal was stained for sympathetic innervation and NGF. After blocking, tissues were rinsed three times for fifteen minutes in unsterile, filtered PBS.

To stain for GDNF and sympathetic innervation, tissues were placed in a 1:500 dilution of sheep anti-GDNF primary antibody and 1:500 dilution of rabbit anti-tyrosine hydroxylase. Tissues were left in primary antibody solution for five days. Then, tissues were washed three times for fifteen minutes each in unsterile, filtered PBS. Tissues were placed in a 1:500 dilution of unsterile, filtered PBS and Alexa donkey anti-rabbit 568nm secondary antibody and a 1:500 dilution of unsterile, filtered PBS and Alexa donkey anti-sheep 488nm. Tissues were left in the secondary antibody solution overnight. The next day, 200 microliters of 50% PBS and 50% glycerol were placed on a slide along with the tissue. A slide cover was placed and sealed with rubber cement followed by clear nail polish to prevent leaks.

To stain for NGF and sympathetic innervation, tissues were placed in a 1:500 dilution of sheep anti-NGF primary antibody and 1:500 dilution of rabbit anti-tyrosine hydroxylase. Tissues were left in primary antibody solution for five days. Then, tissues were washed three times for fifteen minutes each in unsterile, filtered PBS. Tissues were placed in a 1:500 dilution of unsterile, filtered PBS and Alexa donkey anti-rabbit 568nm secondary antibody and a 1:500 dilution of sterile, filtered PBS and Alexa donkey anti-sheep 488nm. Tissues were left in the secondary antibody solution overnight. The next day, 200 microliters of 50% PBS and 50% glycerol were placed on a slide along with the tissue. A slide cover was placed and sealed with rubber cement followed by clear nail polish to prevent leaks.

V. Confocal Imagery

All tissues were imaged using a Nikon C2 confocal at 20x magnitude. Two sections of artery were photographed for each tissue. Each photograph consisted of a maximum intensity projection (MIP) of a Z-stack that spanned the depth of the vessel. TIFS were exported into ImageJ software. Calculations revealed that 1 μm= 0.8 pixels in ImageJ. 576 $pixel^2$ grids were placed on each image and density of sympathetic innervation was quantified based on how many times sympathetic fibers crossed the grid squares (Rebimbas-Cohen, 2005). In order to be counted as a grid crossing, the fiber had to be continuous across the grid square. Since arterial diameter differed throughout the tissues, density was standardized to number of grid crossings per 900μm^2.

VI. Statistical Analysis of Sympathetic Innervation Density

Statistical significance for density of sympathetic innervation was assessed using two-tailed T-tests assuming unequal variance. Significance was set at $p<0.05$.

Results

Exercise had no effect on density of sympathetic innervation

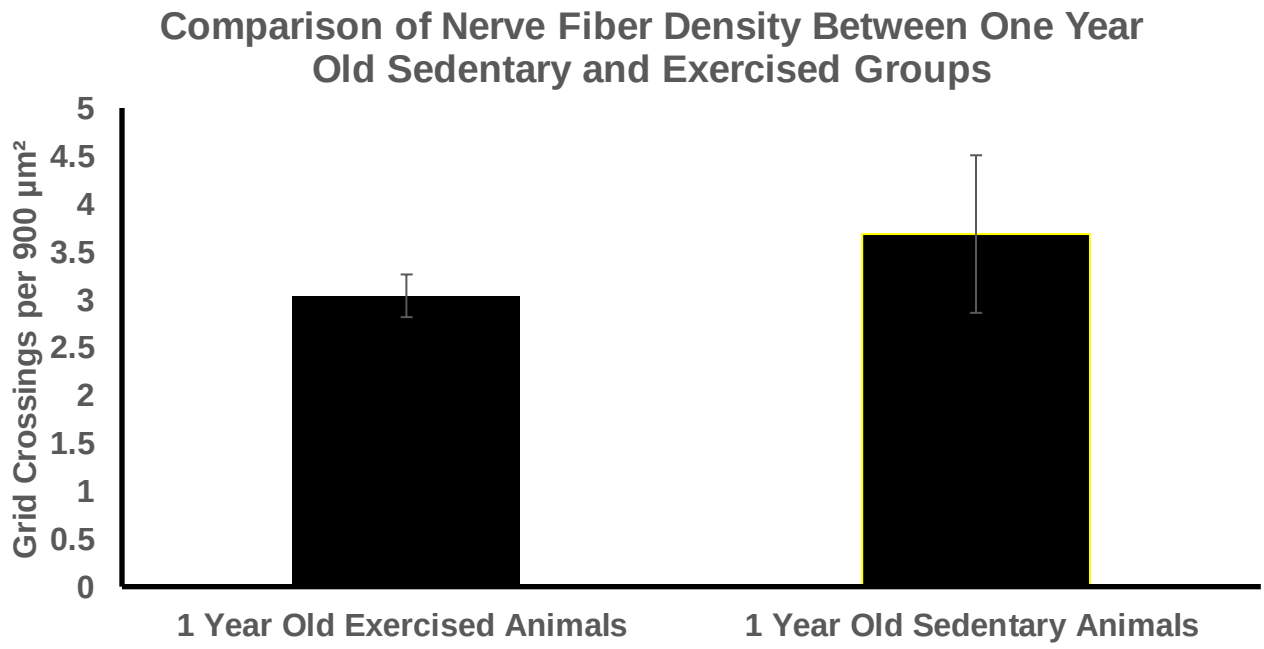

Figure 1: Density of sympathetic innervation was not significantly different between one year old exercised and one-year-old sedentary animals. One-year-old exercised animal tissues (n=20) were compared against one-year-old sedentary animal tissues (n=9) resulting in $p>0.05$.

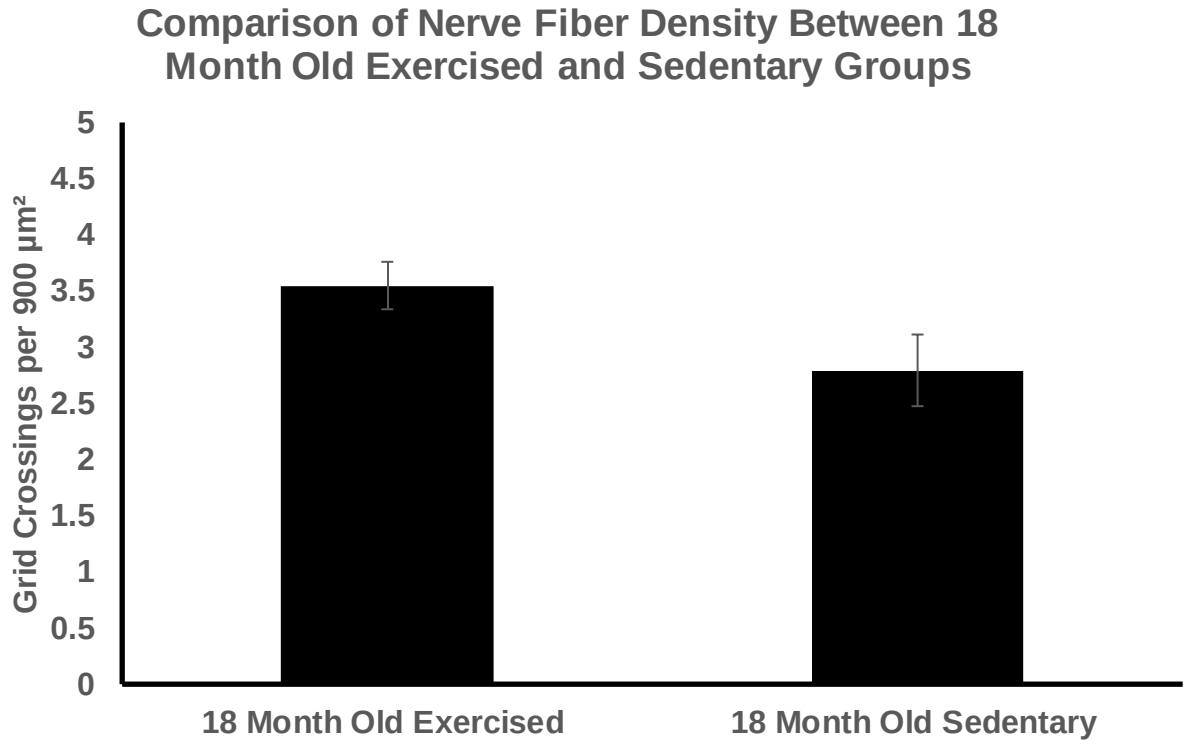

Figure 2: Density of sympathetic innervation was not significantly different between 18-month-old exercised and 18-month-old sedentary animals. 18-month-old exercised animal tissues (n=17) were compared against 18-month-old sedentary animal tissues (n=20) resulting in $p>0.05$.

Density of sympathetic innervation does not change significantly between 4 weeks and one year of age

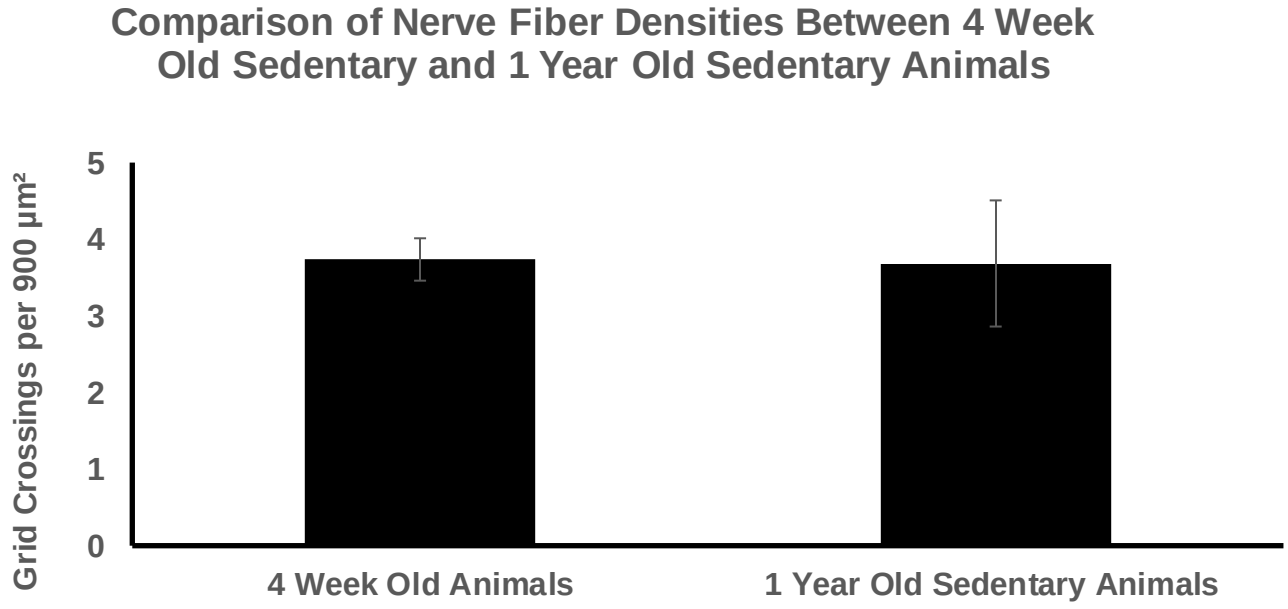

Figure 3: Density of sympathetic innervation was not significantly different between four-week-old sedentary animals and one-year-old sedentary animals. 4-week-old animal tissues (n=24) were compared against one-year-old sedentary animal tissues (n=9) resulting in $p>0.05$.

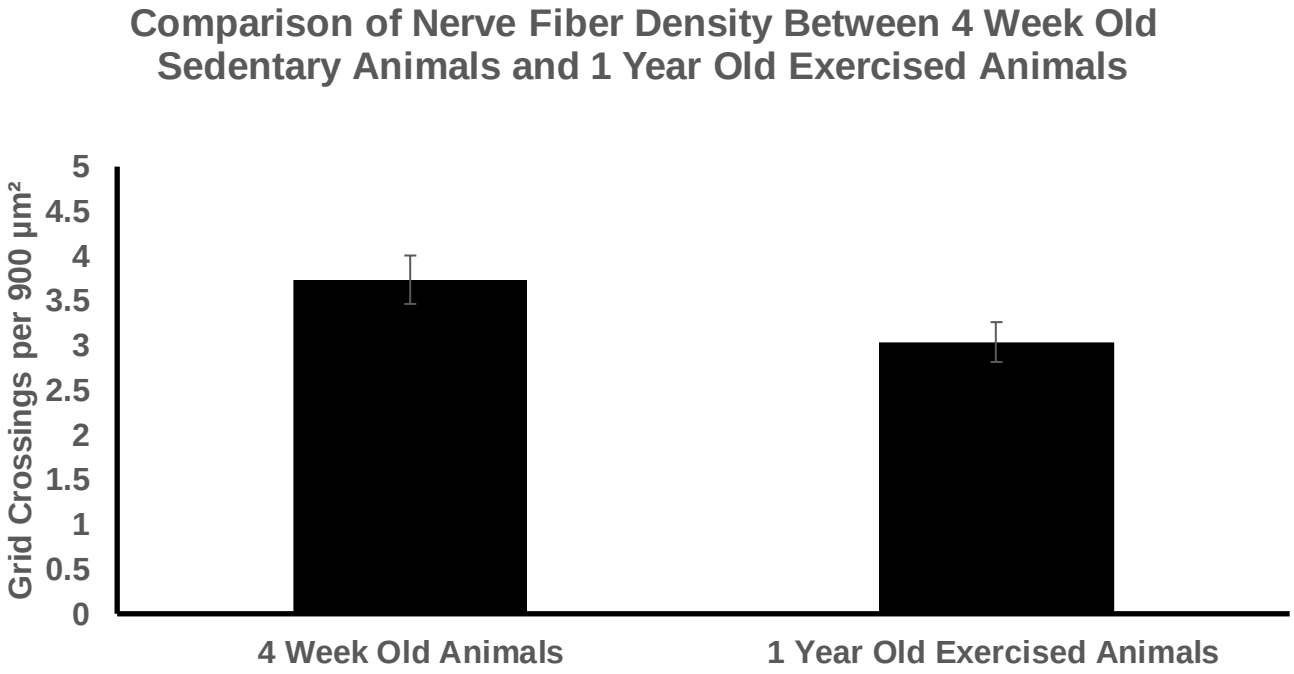

Figure 4: Density of sympathetic innervation was not significantly different between four-week-old sedentary animals and one-year-old exercised animals. Four-week-old sedentary animal tissues (n=24) were compared against one-year-old exercised animal tissues (n=20) resulting in $p>0.05$.

Density of sympathetic innervation was not significantly different between four-week-old animals and 18-month-old exercised animals

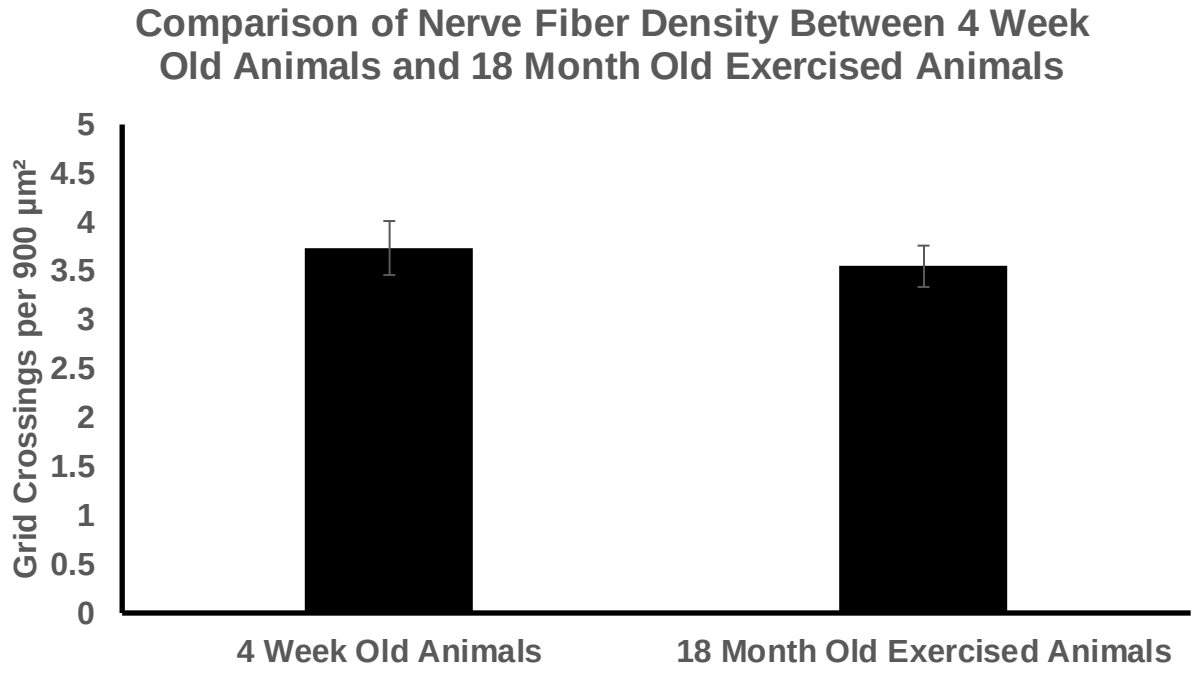

Figure 5: Density of sympathetic innervation was not significantly different between four-week-old sedentary animals and 18-month-old exercised animals. Four-week-old sedentary animal tissues (n=24) were compared against 18-month-old exercised animal tissues (n=17) resulting in $p>0.05$.

Density of sympathetic innervation decreased significantly in 18-month-old sedentary animals

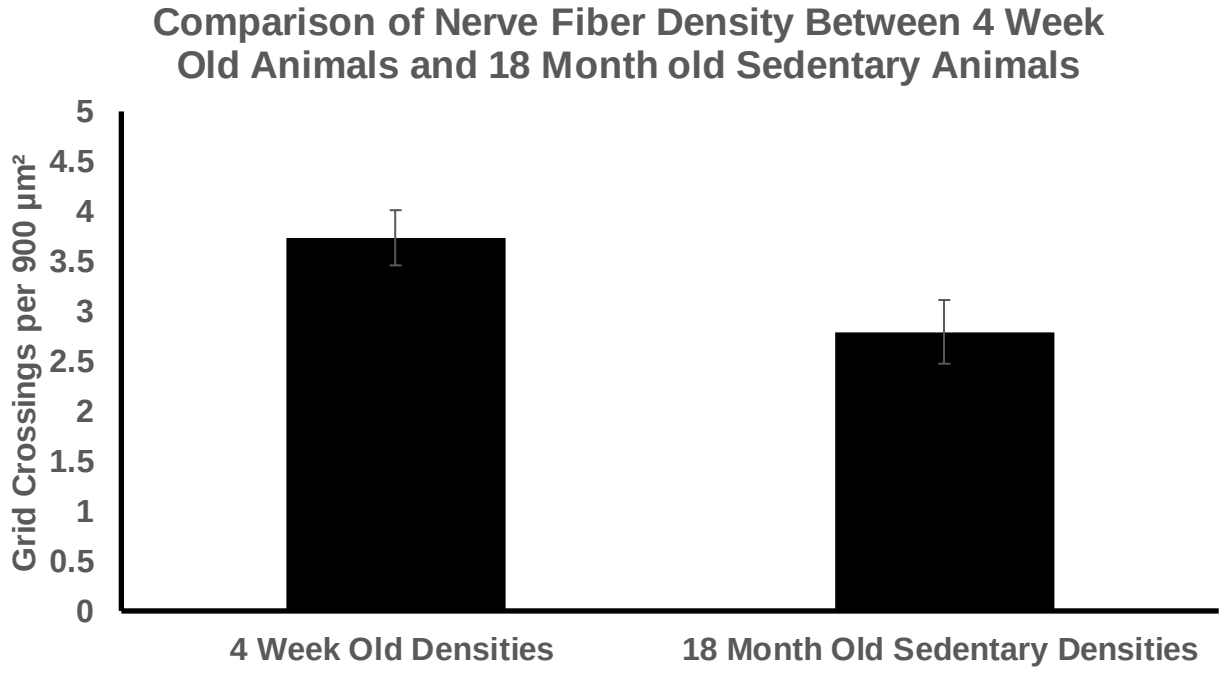

Figure 6: Density of sympathetic innervation was significantly higher in four-week-old animals than in 18-month-old sedentary animals. Four-week-old animal tissues (n-24) were compared against 18-month-old sedentary animal tissues (n=20) resulting in p=0.0313, indicating statistical significance.

GDNF does not appear to be localized within sympathetic neurons

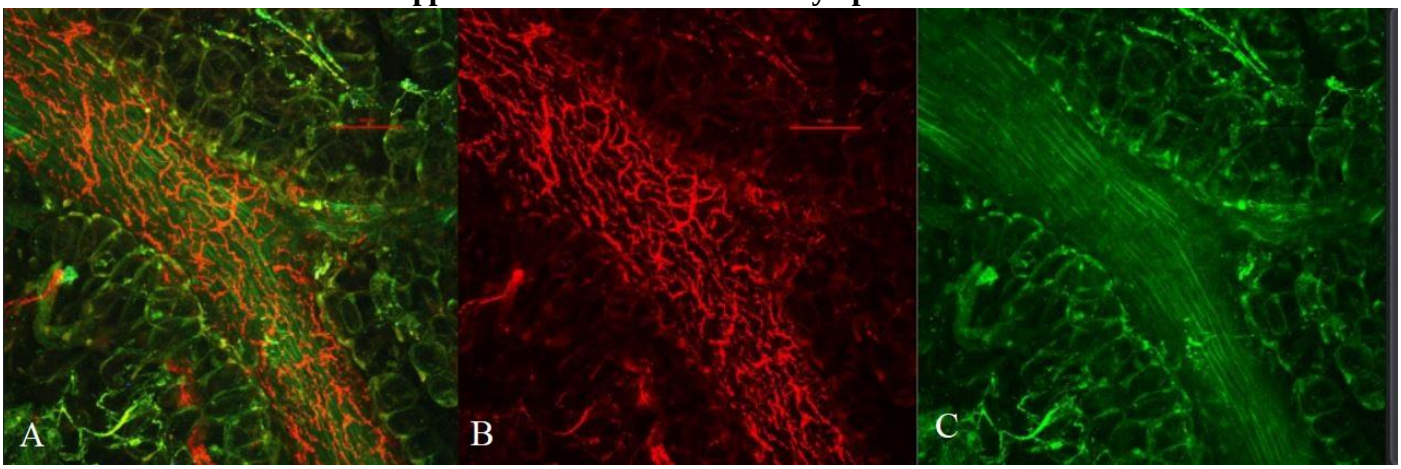

Figure 7: GDNF does not appear to be localized within sympathetic neurons. Panel A shows positive staining for sympathetic innervation in red and GDNF in green on the wall of an artery. Panel B shows just the red channel. Panel C shows just the green channel. If GDNF were localized within sympathetic innervation, there would be a similar pattern as appears in red. Panel C shows GDNF outside the vessel wall and some green within the wall, but GDNF does not appear to be localized within sympathetic neurons because it does not display a similar pattern as the red sympathetic innervation.

NGF does not appear to be localized within sympathetic neurons

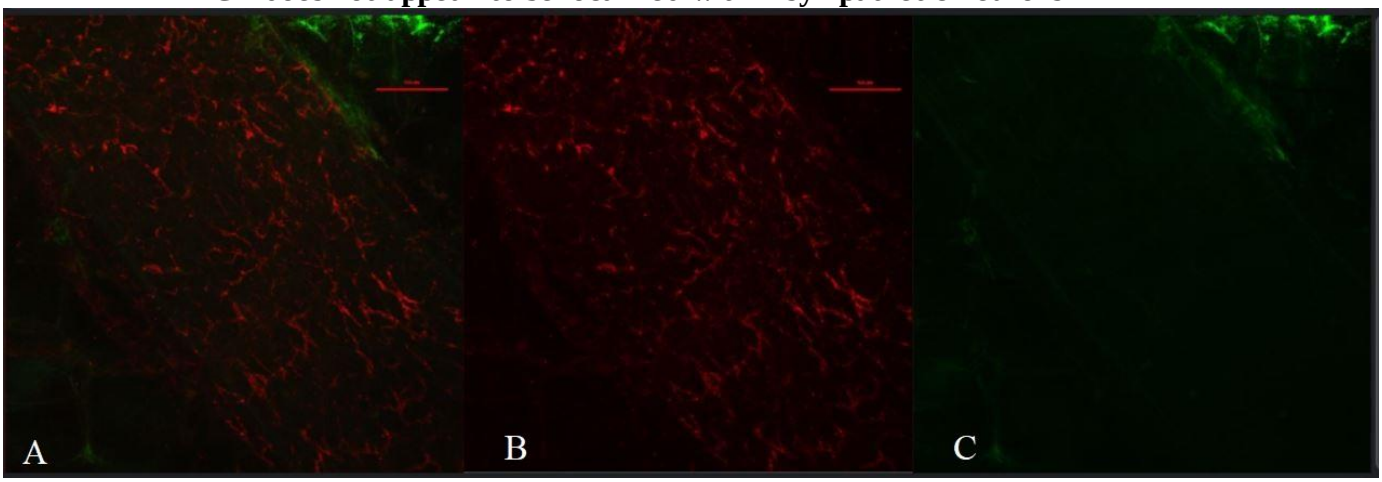

Figure 8: NGF does not appear to be localized within sympathetic neurons. Panel A shows positive staining for sympathetic innervation in red and NGF in green on the wall of an artery. Panel B shows just the red channel. Panel C shows just the green channel. If NGF were localized within sympathetic innervation, there would be a similar pattern as appears in red. Panel C shows a small amount of NGF outside the vessel wall, but none within the wall and none within sympathetic innervation.

NGF appears to be localized around adipocytes

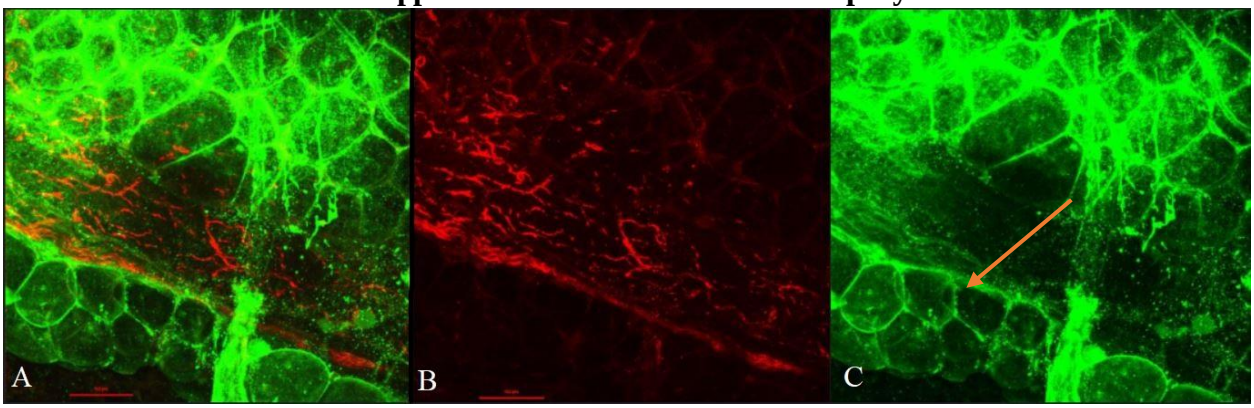

Figure 9: NGF appears to be localized around adipocytes. Panel A shows NGF staining in green and sympathetic innervation in red. Panel B shows just sympathetic innervation in red. Panel C shows only NGF staining. The orange arrow points to an adipocyte outlined in green, suggesting localization of NGF with fat.

GDNF appears to be localized around adipocytes

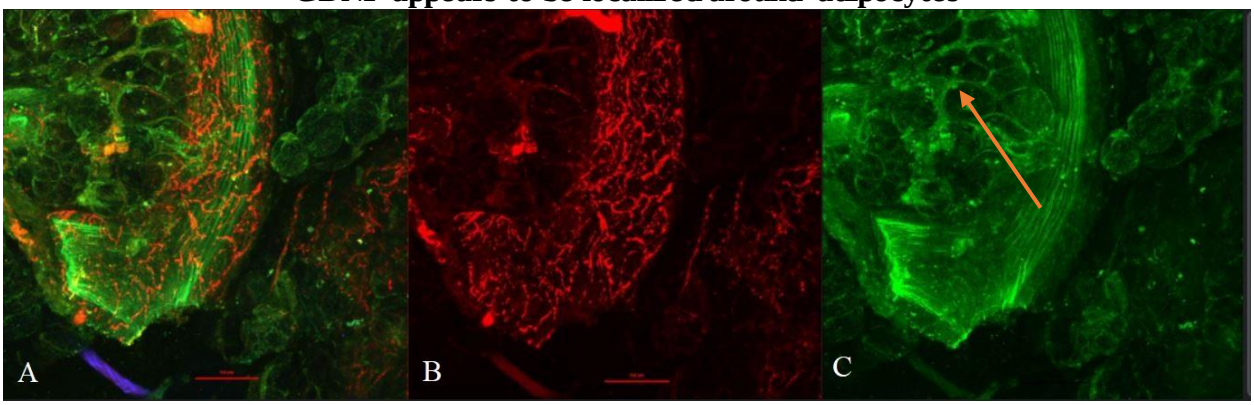

Figure 10: GDNF appears to be localized around adipocytes. Panel A shows GDNF staining in green and sympathetic innervation in red. Panel B shows just sympathetic innervation in red. Panel C shows GDNF staining. The orange arrow points to an adipocyte outlined in green, suggesting localization of GDNF with fat.

Some degree of autofluorescence and background staining occurs in vessel walls

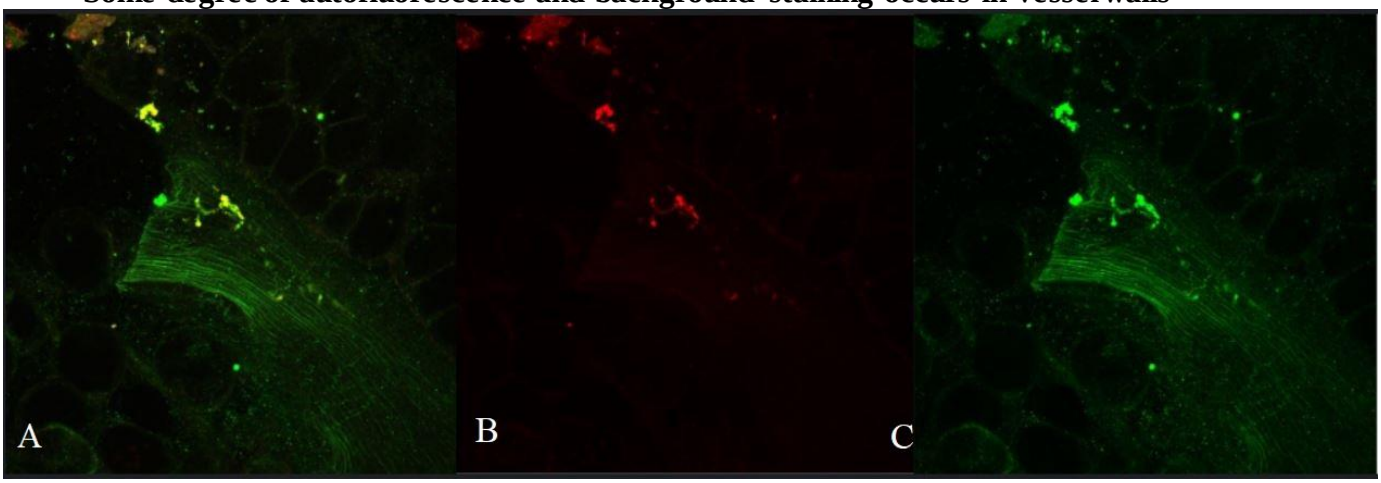

Figure 11: Some degree of autofluorescence and background staining occurs in vessel walls. Panel A shows fluorescence in a vessel that was not stained with primary antibodies against NGF, GDNF, or sympathetic

innervation. Some degree of staining is occurring. Panel B shows just the red channel, where there is no clear pattern of fluorescence. Panel C shows just the green channel, where fluorescence occurs within the vessel wall.

Blood pressure analysis

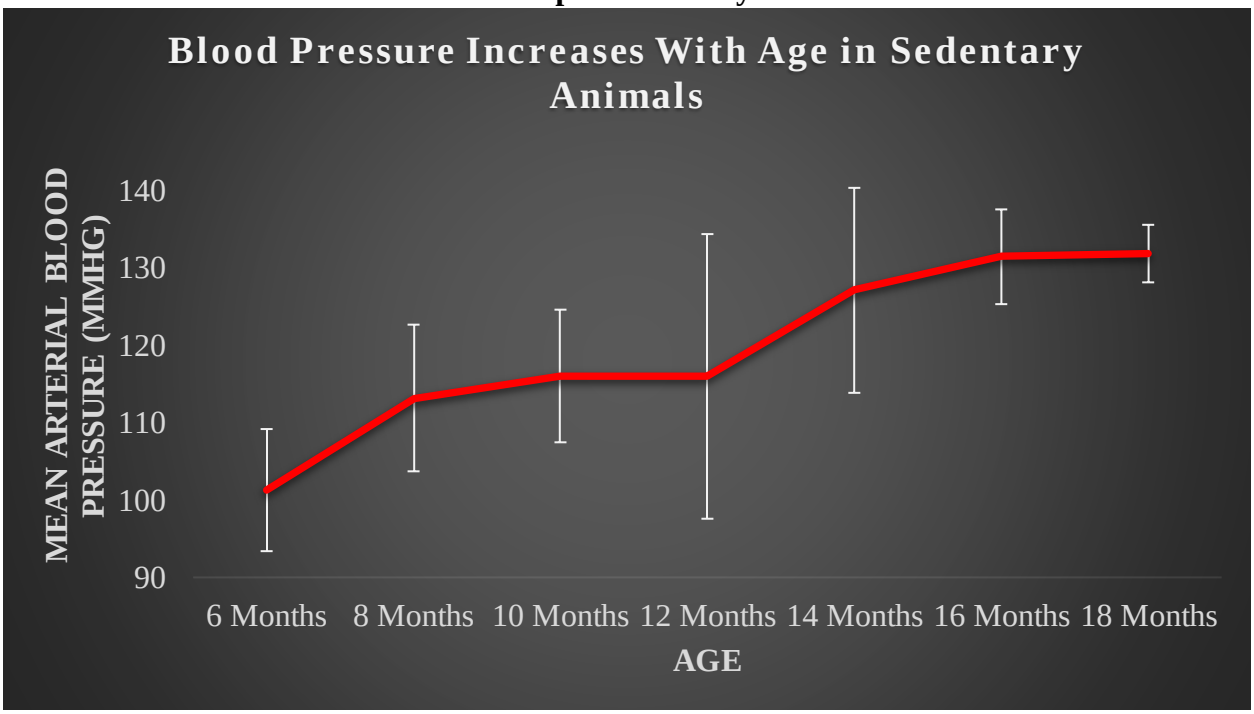

Figure 12: Blood pressure increases with age in sedentary animals. From 6 months of age to 18 months of age, mean arterial blood pressure increased significantly in sedentary animals.

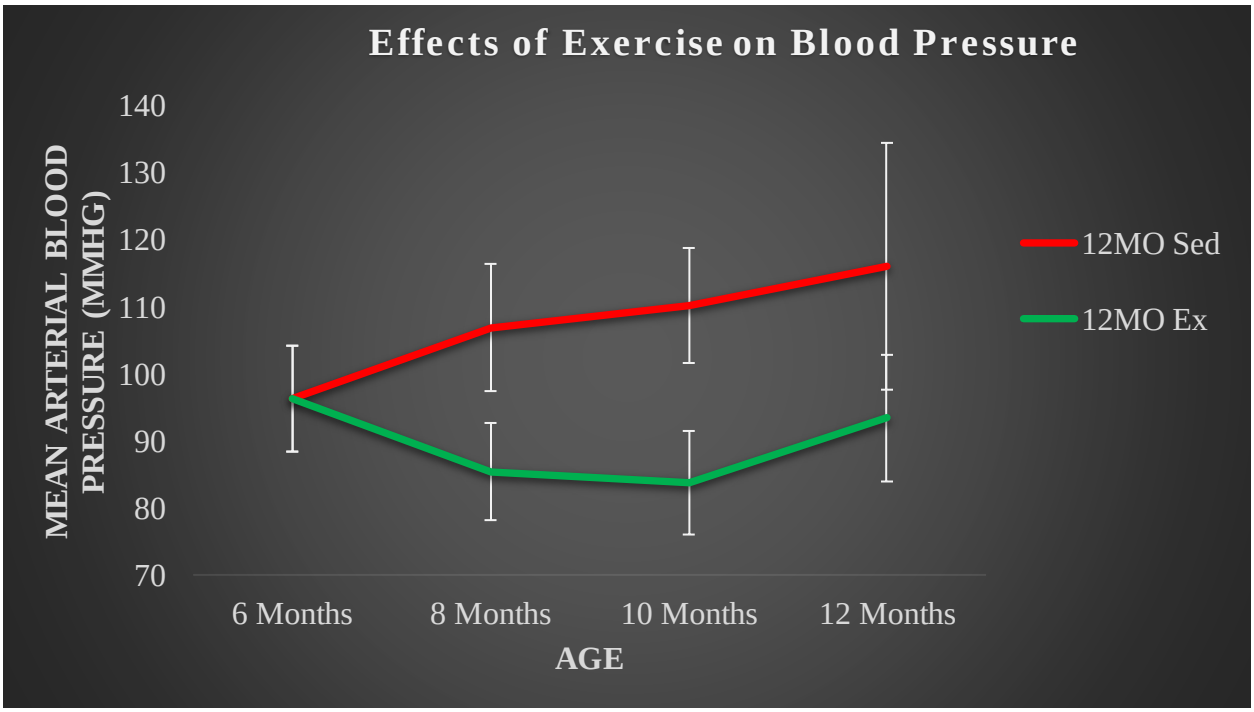

Figure 13: Effects of exercise on blood pressure from 6 months of age to 12 months of age. From 6 months of age to 12 months of age, mean arterial blood pressure was significantly less in exercised animals than in sedentary animals.

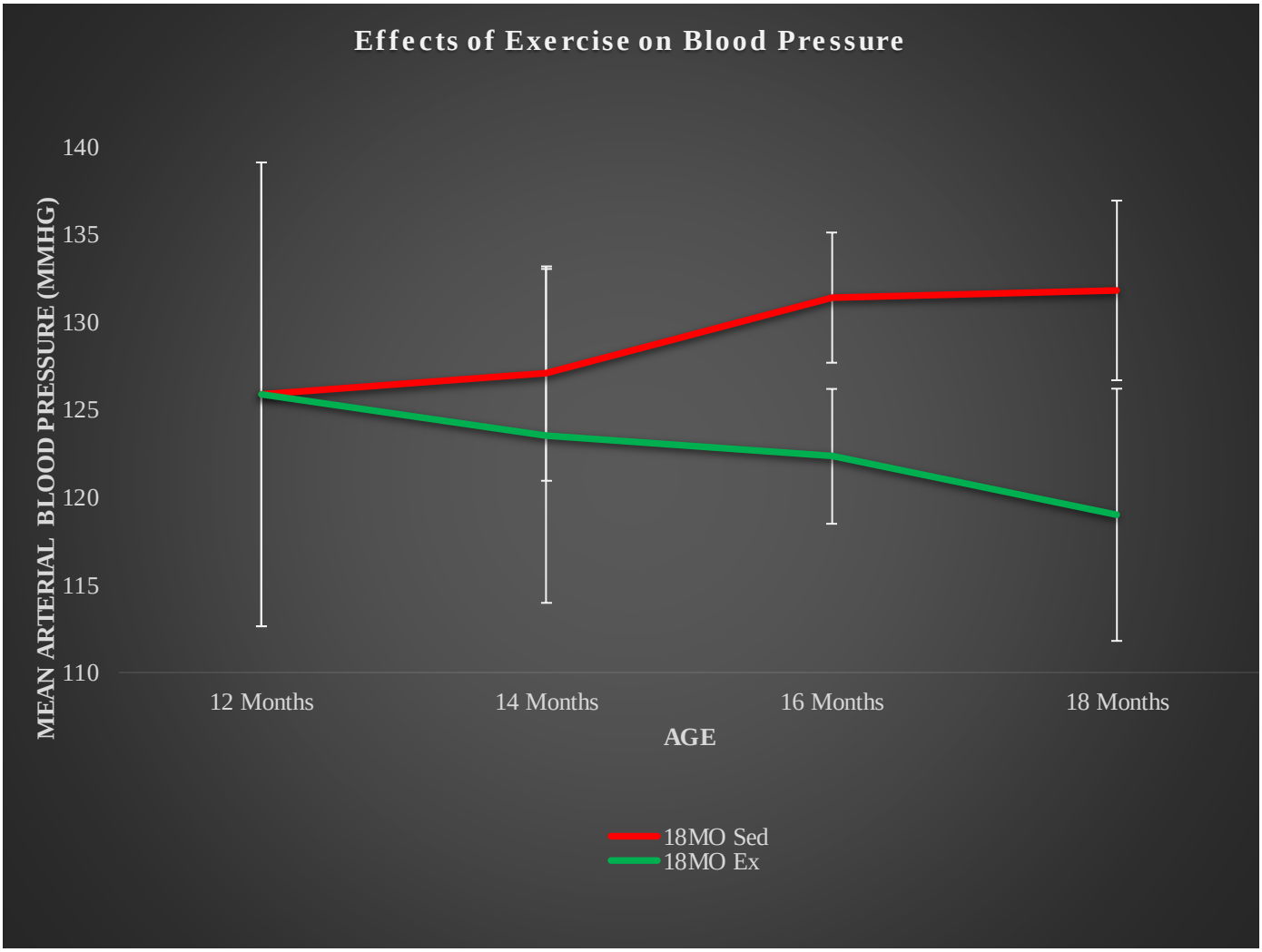

Figure 13: Effects of exercise on blood pressure from 12 months of age to 18 months of age. From 12 months of age to 18 months of age, mean arterial blood pressure was significantly higher in sedentary animals than in exercised animals.

Gross Observations

Fat content around mesenteric artery and vein appeared to change with age. Vessels of older animals had a much greater amount of fat surrounding them, while young animal vessels had much less surrounding fat. Diameter of vessels also appeared to increase with age. Weight also steadily increased as the animals got older.

Discussion

Density of sympathetic innervation was significantly higher in the four-week-old young animals than in the 18-month-old sedentary animals. Exercise seemed to block this effect, as there was no significant difference between density of innervation in 18-month-old exercised animals and four-week-old young animals. These results do not fit the initial hypothesis, which suggested that sympathetic innervation would decrease with exercise. Blood pressure increased with age, and sedentary rats had higher blood pressure than rats who exercised. Since exercise has been shown to decrease hypertension, other mechanisms besides sympathetic innervation may be responsible for the observed reversal of high blood pressure.

Results also do not fit the aging hypothesis, which predicted that sympathetic innervation density would increase with age and possibly contribute to high blood pressure. The four-week-old animals had a mean innervation density of 3.735 ± 0.28 grid crossings per $900\mu m^2$ while the 18-month old animals had a mean innervation density of 2.793 ± 0.32 grid crossings per $900\mu m^2$ ($p=0.031$). Since blood pressure did increase with age for all aging studies, other mechanisms could be responsible for the observed increase in high blood pressure.

Sensory innervation of vasculature has been shown to be associated with lower blood pressure. Sensory innervation utilizes calcium gene-related peptide (CGRP) as a neurotransmitter. CGRP is a potent vasodilator (Brain et al, 1985). Thus, density of sensory innervation and innervation pattern should be examined as a possible mechanism behind changes in blood pressure. It could be that changes in sympathetic innervation density occur alongside changes in sensory innervation density, resulting in an overall increase in blood pressure over time. Since sensory innervation dilates vessels, a decrease in the density of sensory innervation might lead to less dilation, and therefore higher blood pressure. In the future, tissues from these aging studies will be

re-stained and reanalyzed to reveal how the density of sensory innervation changes with age and exercise.

The controls without primary antibody revealed some degree of either autofluorescence or nonspecific staining by secondary antibodies. The same secondary antibody was used for both NGF and GDNF staining. The green fluorescence appearing within vessel walls in the no primary antibody controls could suggest that the secondary antibody sticks to collagen or elastin fibers in arterial walls. To reveal whether this is autofluorescence or nonspecific binding of secondary antibody, a tissue without any antibodies must be imaged using the confocal. A tissue from each age group should be fixed without antibodies and imaged.

Confocal imagery revealed some important pieces of information regarding localization. First, it did not appear that GDNF or NGF were localized within sympathetic fibers. This trend was observed throughout the experiment consistently in each age group and did not differ noticeably with exercise. This suggests that sympathetic innervation itself may not produce NGF and GDNF. Since tissues stained positively for NGF and GDNF, it is possible that tissues around the blood vessels (possibly adipose tissue or smooth muscle tissue) could produce the neurotrophic factors crucial to maintain sympathetic innervation. Though arterial walls appeared to contain NGF and GDNF, it is not clear whether this occurred within smooth muscle cells or whether it was the result of autofluorescence or nonspecific staining. In the future, tissues should be stained with antibodies against smooth muscle cells to reveal localization with neurotrophic factors.

Both GDNF and NGF appear to be localized around adipocytes. It has been shown that NGF is secreted and produced in white adipose tissue (Peeraully et al, 2004). The role of GDNF in adipocytes is not clear. Fat surrounds mesenteric artery and vein, and amount of fat within the mesentery appeared to increase with age. Thus, activity of NGF and GDNF within adipocytes

could affect changes in blood pressure over time. Sex has been shown to affect storage and metabolism in fat (Blaak, 2001). Thus, differences in sex may affect levels of NGF and GDNF surrounding the vasculature. Another possible explanation for specific staining around fat could be that fat cells could possess antigen sites structurally similar to NGF and GDNF. This could be tested by isolating antigens on the cell membrane surface and analyzing and structural similarities with NGF and GDNF.

Future studies will further reveal the role of NGF, GDNF, and innervation patterns in arterial constriction. More age groups will be introduced to develop a complete picture of how sympathetic innervation density changes over a rat lifespan. Tissues from 6-month-old sedentary and exercised rats have been collected. These tissues must be processed and imaged. Rats maintained from 18 months old to two years old are currently being aged. Once this aging period ends, tissues will be collected and processed for confocal imaging. All tissues that were stained for sympathetic innervation will be re-stained for sensory innervation density. Viewing simultaneous changes in both these innervation patterns would further reveal how innervation of arteries changes over time and with exercise. Controls without primary antibody and controls with no antibody will be collected and imaged for each future age group.

Historically, these studies have been conducted using only male rats. Moving forward, studies will also be conducted with female rats to reveal possible differences in blood pressure regulation. Tissues from female rats have already been collected and will be processed for confocal imagery.

Tail arteries have been collected from all rats throughout the aging studies. NGF protein content and GDNF protein content will be quantified using enzyme-linked immunosorbent assay. Assessing how levels of neurotrophic factors within arteries change alongside blood pressure could

further reveal how these factors are involved in blood pressure regulation. When all aging studies are complete, a more complex statistical analysis called ANOVA (analysis of variance) and post hoc tests should be conducted to compare subgroups to each other. These future studies will deepen the understanding of interactions between neurotrophic factors, innervation density, and changes in blood pressure.

Conclusion

More studies must be conducted to reveal the interplay between neurotrophic factors and innervation densities in blood pressure. Since density of sympathetic innervation decreases from four weeks of age to 18 months of age, the physiological consequences of this decrease must be investigated. Exercise blocked this decrease and allowed arteries to maintain a youthful innervation pattern. Revealing the role of neurotrophic factors in regulating blood pressure could reveal therapeutic targets for intervention to combat conditions like hypertension and mediate the harmful precursors to cardiovascular disease.

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