Published in final edited form as:

Psychosom Med. 2017 January ; 79(1): 59–70. doi:10.1097/PSY.0000000000000360.

Structural Remodeling of Sympathetic Innervation in Atherosclerotic Blood Vessels: Role of Atherosclerotic Disease Progression and Chronic Social Stress

Crystal M. Noller, Ph.D.¹, Armando J. Mendez, Ph.D.², Angela Szeto, Ph.D.¹, Marcia Boulina, Ph.D.², Maria M. Llabre, Ph.D.¹, Julia Zaias, D.V.M., Ph.D.³, Neil Schneiderman, Ph.D.¹, and Philip M. McCabe, Ph.D.¹

¹Department of Psychology, University of Miami, Coral Gables, FL 33124

²Diabetes Research Institute, Department of Medicine, University of Miami Miller School of Medicine, Miami, FL 33136

³Department of Pathology, University of Miami Miller School of Medicine, Miami, FL 33136

Abstract

Objective—The sympathetic nervous system (SNS) can undergo dramatic structural plasticity in response to behavioral factors and/or the presence of disease, leading to SNS hyperinnervation of peripheral tissues. The SNS has been proposed as an important mediator between stressful behavior and the progression of atherosclerosis in the vasculature. The present study examined whether structural remodeling of the SNS occurs in the vasculature in a genetically hyperlipidemic animal model of atherosclerosis, the WHHL rabbit (relative to normolipidemic NZW rabbits), and whether SNS plasticity is driven by the progression of disease and/or by stressful social behavior.

Methods—WHHL and NZW rabbits were assigned to an unstable or stable social environment for 4 months. Aortic atherosclerosis was assessed and SNS aortic innervation quantified using immunofluorescent microscopy.

Results—Numerous SNS varicosities were observed throughout the aorta in WHHLs and NZWs, extending into the vascular media and intima, an innervation pattern not previously reported. WHHLs exhibited significantly greater innervation than NZWs ($F(1,41)=55.3$, $p<0.001$), with extensive innervation of the atherosclerotic neointima. The innervation density was highly correlated with the extent of disease in the WHHLs ($r(21)=0.855$, $p<0.001$). Social environment did not influence innervation in NZWs (aortic arch: $p=0.078$, thoracic aorta: $p=0.34$) or WHHLs (arch: $p=0.97$, thoracic: $p=0.61$).

Conclusions—The findings suggest that hyperinnervation is driven largely by the progression of disease rather than social environment. SNS innervation patterns observed in atherosclerotic

Submitting and Corresponding Author: Philip McCabe, Ph.D., Department of Psychology, University of Miami, P.O. Box 248185, Coral Gables, FL 33124, pmccabe@miami.edu, Phone: 1-305-284-2814, Fax: 1-305-284-3402.

Authors report no conflicts of interest.

Conflicts of Interest and Sources of Funding:

human and mouse aortas were consistent with the rabbit, suggesting that SNS hyperinnervation of the diseased vessel wall is a general feature across mammalian species.

Keywords

Sympathetic; Innervation; Atherosclerosis; Remodeling; WHHL; Social Environment

Introduction

Behavioral factors influence the progression of atherosclerosis, a vascular inflammatory disorder underlying Coronary Heart Disease (CHD). Animals exposed to unstable social conditions develop atherosclerosis at an accelerated rate compared to animals in a stable social environment (1, 2). An unstable environment leads to increased agonistic/stressful behavior (2–4), chronic sympathetic nervous system (SNS) activation (3–5), and increased cardiovascular reactivity to stressful stimuli (5). Chronic administration of β -adrenergic antagonists prevents this behaviorally-related atherosclerosis, suggesting that the SNS plays an important role in the progression of disease (6), although the mechanism by which the SNS influences disease has not been established.

The SNS also has been implicated in viral disease progression, and a series of studies has provided a novel alternative to understanding the putative mechanisms by which the SNS influences pathophysiology (7–10). Adult rhesus macaques assigned to an unstable social environment exhibited increased SNS varicosities within axillary lymph nodes relative to monkeys maintained in a stable environment. This effect was pronounced in the extrafollicular regions of the lymph node paracortex that contain macrophages and T-lymphocytes, and was linked to an increased transcription of Nerve Growth Factor (NGF) (8). It has long been known that NGF plays a crucial role in the development of the SNS, and is required for SNS neuron survival and axonal growth (11). The functional consequences of this hyperinnervation included impaired type-1 interferon response to viral infection and an increased replication of the simian immunodeficiency virus (SIV). These investigators also demonstrated that the enhanced SIV replication occurred preferentially in the vicinity of catecholamine nerve varicosities (10). Thus, chronic social stress appears to be related to structural remodeling of SNS innervation via NGF from phagocytes/immune cells, which drives pathophysiologic processes through the release of catecholamines. Since atherosclerosis is a disease characterized by inflammation and infiltration of immune cells into the vessel wall, it is possible that SNS hyperinnervation of vascular tissue may similarly drive disease progression.

SNS hyperinnervation has also been observed in the heart following ischemia and myocardial infarction (12). This SNS sprouting occurred in regions containing accumulations of macrophages and myofibroblasts, and both cell types expressed increased NGF in regions where they were in proximity to SNS fibers (12). In a separate experiment, following pharmacological depletion of cardiac macrophages, NGF expression and SNS innervation density were reduced to control levels (13). Taken together, these studies suggest that macrophage-derived NGF is responsible for SNS hyperinnervation in the diseased heart.

In light of this SNS plasticity in lymph nodes and the heart, the current study examined whether structural remodeling of the SNS occurs in the vasculature in an animal model of atherosclerosis, the Watanabe Heritable Hyperlipidemic Rabbit (WHHL), compared to a normolipidemic control (New Zealand White Rabbit; NZW). WHHLs have a spontaneous genetic defect in cholesterol clearance, exhibit extremely elevated serum cholesterol and triglycerides from birth, and develop severe aortic atherosclerosis over the first 7 months of life (14, 15). We evaluated whether social environment alters vascular innervation patterns in the presence or absence of atherosclerotic disease. In the presence of SNS hyperinnervation, SNS activation due to social stress could lead to more extensive and severe disease. Alternatively, we also assessed whether the extent of disease, independent of social environment, drives SNS innervation. In this case, monocyte/lymphocytes infiltration into the neointima may induce SNS hyperinnervation via the release of neurotrophins such as NGF. In either case, SNS plasticity and remodeling in the vasculature could represent an important mechanism linking the SNS to the progression of atherosclerotic disease.

Methods

Experimental Subjects

Twenty-two male WHHL and 24 male NZW rabbits (2.5–3.0 months old, 1.5–2.0 kg) were obtained from Covance Research Products, Inc. (Denver, PA). Animals were housed in individual cages (6 sq ft) in a room with artificially controlled temperature (22–24 °C) on a 12 h light/dark cycle (lights on at 07:00 h). All experiments and aftercare handling were conducted during the light portion of this cycle. Rabbits were provided 8 ounces of standard rabbit chow (Purina; 2.5% total fat; 0% cholesterol) daily. Water was provided ad libitum. Histological evaluation of aortic tissues from mice and humans was also performed for comparison to rabbits. Four male ApoE^(-/-) mice and four C57Bl/6 mice, 11–12 weeks of age, weighing 22–24 g) were purchased from Jackson Laboratory (Bar Harbor, Maine). At 32 weeks of age mice were euthanized by carbon dioxide narcosis. The procedures for the care, use and euthanasia of experimental animals followed the protocols and regulations set forth by the Animal Care and Use Committee of the University of Miami and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. De-identified human aortic tissue was obtained from 4 organ donors that consented to provide tissue for research use through the University of Miami Organ Procurement Office, with prior approval from the University of Miami Human Subjects Institutional Review Board and in accordance with the Helsinki Declaration.

Behavioral Procedures

After acclimation procedures, rabbits were assigned to either unstable or stable social conditions. In the unstable groups, animals were paired together for 4 hours per day. Each week the pairings were switched within the groups, requiring rabbits to continually reestablish dominant-subordinate relationships. Because the pairings occurred in the home cages of the animals, each week one half of the rabbits remained in their home cage and one half of the rabbits were intruders. The home-intruder relationship of animals was counterbalanced across the duration of the entire study. In the stable groups, animals were paired daily for the same 4-hour time period as the unstable group. Unlike the unstable

group, the stable animals remained in the same pairings for the entire study. As in the other group, the home-intruder relationship was counterbalanced across the study. The stable group consisted of littermate pairs while the unstable group contained non-littermates. The size of resultant social groups were as follows: within the WHHL group, 12 animals were assigned to an unstable environment and 10 animals to a stable environment, and within the NZW group, 14 animals to an unstable environment and 10 animals to a stable environment.

When the rabbits were 3 months old, the social pairings were initiated and continued daily until the animals were 7 months old. The occurrence of specific behaviors was scored during week 1, at midpoint and the final week of behavioral pairing. Behaviors were assessed for each animal during the first 10 minutes of the social pairing for that day. Behaviors were classified into the following major categories: agonistic behavior, affiliative behavior, other nonagonistic behavior, and inactivity (16, 17). Agonistic behaviors included approach behavior, chasing, mounting, copulation, pawing, marking, enuresis, biting, combat, immobility or freezing, escape, escape mount, squealing, and thumping. Affiliative behaviors included grooming the cagemate, sniffing the cagemate, nuzzling, and lying adjacent to the cagemate (17). Other nonagonistic behaviors included self-grooming, cage exploration, and drinking or eating. Inactivity consisted of passive rest, i.e., stretched out in a relaxed posture or sitting quietly in the cage not in proximity to the cage mate. Because these behaviors consist of behavioral acts, which are momentary, and behavioral states, which persist over time, all behavioral activity was scored as the percentage of time that was spent in that behavior during the observation period. The individual behavioral scores were then combined into the 4 main categories (i.e., agonistic behavior, affiliative behavior, other nonagonistic behavior, and inactivity). The total percent time in each behavioral category for each animal was then calculated for the initial week of pairing, the midpoint week of the study, and the last week of pairing. The behavioral scores for these 4 categories represented in Table 1 were the average of these three time points.

Measurement of Atherosclerosis

After euthanasia (by i.v. injection of 90 mg/kg pentobarbital and 12 mg/kg phenytoin sodium), the entire aorta was removed via a midline thoracic and abdominal incision, and the tissue was placed in a 10% solution of buffered formalin. Samples were coded, and all histomorphometric procedures were performed in a blind fashion by a trained pathologist. The aorta was dissected longitudinally, and *en face*, the two-dimensional area of each aortic lesion from each animal was calculated by multiplying the length and width of each lesion in mm. The area of all of the lesions in the aortic arch and thoracic aorta for each animal was summed for the calculation of total lesion area (mm²). For the entire extent of the aorta, the surface area of each lesion or plaque was identified by the border of raised intima and presence of foam cells/fatty streaks. Each lesion was sectioned at the point of maximum height, or midlesion, for cellular analysis. For sectioning, the tissue was paraffin-embedded, sectioned at 5 µm, and stained with hematoxylin and eosin. Aortic lesions were observed under light microscopy and graded according to the American Heart Association histologic classification for atherosclerotic lesions (18).

Immunofluorescent Detection of SNS Varicosities

SNS varicosities were identified by the immunohistochemical co-localization of tyrosine hydroxylase (TH) and synapsin (19–21). Synapsin is a vesicular-associated protein highly expressed in nerve cells, and is concentrated in nerve terminals as well as varicosities (22, 23). Synapsin plays an important role in the regulation of neurotransmitter release from vesicles (24), and therefore it is localized to sites of vesicular accumulation and transmitter release rather than neuronal cell bodies or axons (22). TH is an enzyme involved in the biosynthesis of catecholamines, and is found in all cells that produce catecholamines, including neurons and immune cells (25). The co-localization of TH and synapsin only occur in neurons, and more specifically at neuronal sites of catecholamine release from vesicles (e.g., SNS varicosities).

Three to four mm segments of formalin-fixed aortic tissue was embedded in paraffin blocks. Representative blocks from the aortic arch and thoracic aorta were selected for each rabbit. Using a microtome, 26 consecutive 10µm cross sectional slices were obtained and mounted on glass slides from each representative block. Using the Systematic Random Sampling method (26) every 5th section was selected for immunofluorescent staining, and the last section was used for hematoxylin and eosin staining.

Samples were deparaffinized and antigen retrieval performed using the citrate buffer method (27). Prior to immunostaining, slides were washed with PBS then incubated with blocking solution (PBS containing 1% BSA and 10 % donkey serum). The primary antibodies were chicken anti-tyrosine hydroxylase (1:500 dilution; Millipore, Billerica, MA), guinea pig anti-synapsin for rabbit tissues (1:250 dilution, Synaptic Systems, Germany), rabbit anti-synapsin-1 for mouse tissues (1:100 dilution, Abcam, Cambridge, MA), mouse anti-rabbit macrophage (RAM-11; 1:100 dilution, Dako, Carpinteria, CA), and rat anti-NGF (1:1000 dilution, Promega, Madison, WI). Species matched isotypes controls for the primary antibodies were evaluated for staining specificity (e.g., Figure 2). The TH antibody used in this study has been shown to specifically stain nerve fibers and varicosities in a variety of tissues (19, 21, 28, 29). The specificity of the synapsin antibody was verified by the manufacturer (unpublished data, Synaptic Systems) that showed absence of immunoreactivity in synapsin 1,2 knockout mice relative to wild type mice that exhibited the expected synapsin immunoreactivity.

Antibodies were diluted in blocking buffer and incubated overnight at 4°C. Non-immune immunoglobulins from the same species were used to assess non-specific staining. Slides were rinsed with wash buffer (Biogenix, Fremont, CA) and then incubated with species-specific labeled secondary antibodies from Jackson ImmunoResearch (West Grove, PA) and used at recommended concentrations. For NGF detection the tyramide amplification was used (Perkin Elmer, Waltham, MA). Secondary antibodies were incubated for 2 hours at room temperature followed by rinsing with wash buffer and then PBS. Finally, slides were mounted using ProLong Gold with DAPI (Life Technologies, Grand Island, New York).

Tiled images of entire aortic cross-sections were acquired using an Olympus immunofluorescence microscope [VS120-FL, Center Valley, PA] with a 10x objective lens that resulted in a 640 nm/pixel resolution. High resolution images were acquired using a

Leica SP5 inverted confocal microscope with 20x/0.70 NA and 40x/1.25 NA objective lens. Line averaging was used to improve signal-to-noise ratio and during image acquisition.

Image Analysis and SNS Varicosity Quantification

The *Bio-Formats* plugin of FIJI/Image J (30) was used to import Olympus image files for each channel. Metamorph software (Molecular Devices, Sunnyvale, CA) was utilized to designate intimal and medial regions of interest (ROI) based on the DAPI channel. Figure 1 demonstrates intimal and medial regions on representative WHHL and NZW tiled images. Prior to quantification, the tyrosine hydroxylase and synapsin images were processed separately to subtract background signal using the Gaussian blur filter. Positive signals were binarized and segmented to separate touching objects. The two processed channels were combined to produce an image where varicosities were identified by quantifying the number of objects positively stained for both fluorescent probes using the Analyze Particles and Measure features of the Image J program. Regions stained only for tyrosine hydroxylase or only synapsin were not considered to be sympathetic varicosities.

The SNS innervates the vascular adventitia, where it forms a plexus of branching axons, and norepinephrine is released from structural varicosities situated periodically along the length of catecholaminergic axons (31). Varicosities are often fusiform in shape, typically measure 1–3 μm in diameter and up to 4 μm in length (32). For defining varicosities, a size range of 0.83 – 12.60 μm^2 was used for quantification based on the largest cross sectional length described in previous reports (33, 34). An object the size of one pixel (e.g., 0.643 μm) was considered to be indistinguishable from background noise. Sympathetic innervation density was defined as the total number of varicosities per area (mm^2) of the section, using the average of three slides for each aortic region.

Timeframe of Data Collection

The social pairings and behavioral data were collected over a 4-month period. Subsequently, histopathology and the immunohistochemical procedures/analyses were conducted over 12 months.

Statistical Analyses

The behavioral data were analyzed using a 2 (Rabbit Strain; WHHL vs NZW) x 2 (Social Condition; Unstable vs Stable) analyses of variance (ANOVA) design for each of the four measured behaviors (e.g., Agonistic, Affiliative, Other Non-Agonistic, Inactivity). The total area of atherosclerosis within the aortic arch and the thoracic aorta was examined using a 2 (Rabbit Strain) x 2 (Social Condition) ANOVA. Significant interactions were followed up with group comparisons. SNS innervation was evaluated using a 2 (Rabbit Strain) x 2 (Social Environment) x 2 (Aortic Location) mixed design ANOVA. Within that design, specific comparisons of interest were examined using ANOVA. Preliminary data screening using boxplots within rabbit strains indicated no extreme outliers. Finally, correlational analyses assessed the relationship between the number of SNS varicosities and the intimal area of the aorta within each rabbit strain. Standard 2-tailed tests with p -values <0.05 were used to determine significance.

Results

Behavior

Animals in an unstable social environment exhibited significantly more agonistic behavior relative to animals in a stable environment, $F(1,42) = 16.5$, $p < 0.001$; see Table 1). In contrast, a stable social environment was characterized by increased social affiliative behavior, ($F(1,42) = 28.0$, $p < 0.001$) and other non-agonistic behavior (e.g., eating, drinking, cage exploration ($F(1,42) = 18.2$, $p < 0.001$) compared to the unstable group. There was no significant difference in behavioral inactivity between the social groups, $p = 0.21$. As expected, there was no significant difference in agonistic behavior, affiliative behavior, or behavioral inactivity between WHHL and NZW rabbit strains ($p = 0.36$, 0.24 , and 0.68 , respectively). WHHL rabbits spent more time in other non-agonistic behavior compared to NZW, $F(1,42) = 6.7$, $p = 0.013$. The behavioral findings are consistent with previously published reports from our laboratory (2–4).

Area of Aortic Atherosclerosis

There was a significant interaction in aortic atherosclerosis between rabbit strain and social condition, $F(1,42) = 4.6$, $p = 0.038$. Within the WHHL strain, animals in the unstable group ($481.4 \text{ mm}^2 \pm 88.1$) had a greater area of disease compared to rabbits in the stable group ($217.4 \text{ mm}^2 \pm 91.8$), $F(1,20) = 4.3$, $p = 0.052$, suggesting that social environment influences the progression of atherosclerosis. There was minimal disease in the NZWs, and thus there was no difference in disease between social conditions within this group ($p = 0.17$). Not surprisingly, there was also a main effect of rabbit strain, $F(1,42) = 32.2$, $p < 0.001$, such that WHHLs displayed extensive disease ($361.4 \text{ mm}^2 \pm 68.4$, mean $\pm$ SEM), while as described above, disease in the NZWs was essentially absent ($1.1 \text{ mm}^2 \pm 0.6$).

Assessment of Aortic SNS Innervation

Sympathetic varicosities were observed throughout the aortic arch and thoracic aorta in both NZW and WHHL rabbits (Figure 3). In the normolipidemic NZW animals there were abundant varicosities throughout the vascular media, and in some cases there was sparse labeling extending into the intimal region. In the WHHLs dense varicosity labeling was seen throughout the media, extending into the intima, and with abundant labeling in the atherosclerotic neointima.

Innervation density was quantified in sections in the aortic arch and the thoracic aorta from both rabbit strains. Overall, there was a main effect of aortic location, such that the aortic arch was more densely innervated than the thoracic aorta, $F(1,41) = 13.4$, $p = 0.001$. The three way interaction among rabbit strain, social condition, and aortic location was not significant, $p = 0.50$, however, there was a significant interaction between rabbit strain and aortic location, $F(1,41) = 5.6$, $p = 0.023$. Within the NZW group, there was greater innervation in the aortic arch compared to the thoracic aorta ($F(1,22) = 33.2$, $p < 0.001$), while there was no difference in innervation density between aortic regions in the WHHLs, $p = 0.48$. For this reason, additional analyses were conducted separately for each aortic location. In general, WHHLs exhibited greater innervation density than NZWs, $F(1,41) =$

55.3, $p < 0.001$, and within the aortic arch ($F(1,41) = 11.9$, $p = 0.001$), and the thoracic aorta, ($F(1,42) = 48.2$, $p < 0.001$), as illustrated in Figure 4.

There was no significant interaction between rabbit strain and social condition in either the aortic arch or the thoracic aorta ($p = 0.22$ and 0.98 , respectively; Table 2). As described above, because we found innervation differences between aortic locations within the NZW group, we also analyzed social environment within rabbit strains separately. Within the NZWs, there was no significant difference in innervation density between social conditions in the aortic arch or thoracic aorta ($p = 0.078$ and 0.34 , respectively). Since these animals did not exhibit disease, these data suggest that social environment alone does not alter SNS innervation. Similarly, in WHHLs we did not observe a difference in innervation density between unstable and stable social conditions in the aortic arch or in the thoracic aorta ($p = 0.97$ and 0.61 , respectively).

Whereas innervation density was not influenced by social environment, innervation was related to the extent of atherosclerosis. The number of intimal SNS varicosities increased as neointimal area expanded due to atherosclerotic disease progression. Within the WHHL group, there was a significant relationship between the number of varicosities within the intima and the total intimal area in both the aortic arch ($r(21) = 0.86$, $p < 0.001$) and thoracic aorta ($r(22) = 0.79$, $p < 0.001$; see Figure 5A–D). In contrast, there was no relationship between the number of intimal varicosities and the intimal area for the NZWs, largely due to the fact that they had no disease, $p = 0.22$ and 0.58 for the aortic arch and thoracic aorta locations, respectively. Therefore, because the WHHLs developed significant atherosclerosis relative to the NZWs and exhibited greater aortic innervation density than NZWs, these data suggest that vascular SNS hyperinnervation is driven primarily by disease progression, or hyperlipidemia.

It has been reported that SNS innervation in lymph nodes and the myocardium is affected by locally produced NGF (8, 12). In a preliminary examination in the WHHL, we assessed NGF expression in vascular tissue (Figure 6). The extent of tyrosine hydroxylase labeling from an aortic cross-section in a region of disease showed extensive SNS innervation throughout the vessel wall, including the neointima (Figures 6B & 6C). In addition, high magnification images of the region highlighted in panel 6B (panels 6C–F) illustrated immunostaining of NGF within, and proximal to, macrophages in the vicinity of the TH-labeling. Also observed was the relative absence of TH, macrophage and NGF-labeling within regions of necrosis (seen as dark areas devoid of nuclear staining).

SNS Varicosities in Human and Mouse Aortas

In order to demonstrate that these patterns of vascular innervation were not unique to the rabbit strains studied, we examined mouse and human aortic tissues for the presence of SNS varicosities. Figure 7 illustrates the presence of SNS varicosities within the non-diseased C57Bl/6 mouse (wildtype) aorta and atherosclerotic mouse (ApoE^(-/-)) aorta (panels 7B and 7D, respectively). ApoE^(-/-) mice have a phenotype similar to the WHHL rabbit in that they are dyslipidemic and spontaneously develop aortic atherosclerosis. In the non-diseased wildtype mouse, varicosities are present in the vascular media, while in the ApoE^(-/-) mouse aorta varicosities are present in both the media and atherosclerotic intimal lesions.

Figure 8 shows SNS varicosities within a human aortic section, again demonstrating the presence of innervation throughout the media and within the atherosclerotic neointima. These innervation patterns are consistent with the rabbit aortic data, suggesting that SNS innervation of the diseased and non-disease vessel wall is a general feature across mammalian species.

Discussion

The major finding of this study is that the SNS innervation of the vasculature is structurally remodeled in relation to the progression of atherosclerosis in hyperlipidemic rabbits prone to the development of disease. In animals with atherosclerotic lesions, extensive SNS varicosities were observed within the neo-intima, which is an area rich with migrating monocytic cells and lymphocytes (35). The SNS hyperinnervation paralleled the growth of the neo-intima as disease progressed until the plaque advanced to a severe stage where significant necrosis was observed. This innervation pattern was seen throughout the aortic arch and thoracic aorta. Hyperinnervation of atherosclerotic lesions was also observed in both human and mouse aortas, suggesting that structural SNS remodeling related to vascular disease is a general phenomenon across mammalian species. This represents the first report of SNS innervation of the neo-intima. Throughout the intima and media, the density of SNS innervation was greater in the WHHL rabbits than in the normolipidemic, non-diseased NZWs, suggesting that either the extent of disease or hyperlipidemia drove the innervation differences between rabbit strains.

In non-diseased arteries, the SNS innervation historically has been described as being confined to the arterial adventitial layer, terminating at the border of the adventitia and media (smooth muscle) layers (36, 37), where they form a plexus of branching axons. Norepinephrine is released from structural varicosities situated periodically along the length of catecholaminergic fibers (31). Although SNS varicosities were originally not thought to penetrate into the media but lay at a distance from smooth muscle cells, it has been reported that SNS fibers form neuromuscular contacts with some smooth muscle cells (34). Upon activation of SNS neurons, norepinephrine is released and diffuses through the media and can reach the intimal surface and endothelium (31). There have been no reports in the literature, however, that SNS fibers can extend into the intimal layer of blood vessels. In the current study, non-diseased, normolipidemic NZW rabbits exhibited extensive SNS varicosities throughout the vascular smooth muscle, and in some cases extending all the way to the endothelial cell layer of the intima. This observation is in contrast to most previous reports that described the normal SNS innervation as terminating at the vascular adventitia and adventitia-media border. It is possible that differences in histological methods between the current study (i.e., immunohistochemistry) and previous studies (i.e., formaldehyde or glyoxylic acid to label catecholamines) may account for some of the discrepancies in the reported innervation patterns.

In the present study, SNS varicosities were identified using immunohistochemistry to co-localize TH and synapsin. It has been reported that TH is also expressed in non-neuronal cells such as lymphocytes, macrophages and other immune cells (25, 38). Similarly, it has been reported that synapsin is also found in some non-neuronal cells (e.g., osteoblasts (39)

insulinoma cells (40), epithelial cells (41)), but it has not been reported in vascular or immune cells. In spite of these reports, the literature suggests that we are quantifying SNS varicosities because: 1) SNS varicosities and nerve terminals are the only structures where TH and synapsin are co-localized, 2) the immunohistochemically identified structures in the aortas were within a size documented for nerve terminals and varicosities ($0.83\text{--}12.60\ \mu\text{m}^2$), which are significantly smaller than the area for the typical immune cell ($> 75\ \mu\text{m}^2$), and 3) the labeled structures were not nucleated (as determined by DAPI staining, e.g., Figure 3), and therefore are consistent with varicosities rather than nucleated immune cells.

In the current study, we did not find any evidence that social environment influences the pattern or density of vascular SNS innervation in either WHHLs or NZWs. This is consistent with previous work showing no differences in innervation patterns of small blood vessels in the perivascular region of the lymph nodes as a function of social environment (8). Interestingly, this study also reported that animals in an unstable social environment had greater SNS innervation of the lymph node parenchyma (a region rich in immune cells) than animals in a stable social environment (8). Therefore, it appears that social environment may differentially influence innervation patterns in the lymph node parenchyma as opposed to blood vessels. The inclusion of non-diseased NZW rabbits in our study (that did not exhibit differences in innervation as a function of social environment) suggests that the critical factor in SNS vascular remodeling may be disease progression (i.e., monocytic cell infiltration) rather than social environment. Alternatively, it is also possible that differences in innervation density between social conditions might have been observed at an earlier time point in disease progression (i.e., before extensive disease is seen in animals in both social environments). At 7 months of age the stable social group had enough disease (and therefore enough macrophage infiltration and NGF release) to trigger significant hyperinnervation, such that any differences in innervation due to social environment may have been obscured. Future studies will examine innervation patterns at earlier time points to address this issue.

In the WHHL rabbits, the unstable social group exhibited a significantly greater area of aortic atherosclerosis (as determined by *en face* quantification of the entire aorta) than the stable social group; a finding that we have previously reported (2, 4). In spite of this group difference in disease, the pattern and density of SNS varicosities was very similar between social groups. On the surface this is puzzling, because if innervation density is related to the progression of atherosclerosis, one might expect the group with less disease to have less innervation. A possible explanation for this apparent discrepancy is that the measure we used to quantify atherosclerosis was lesion area for the entire vessel, whereas SNS varicosity measurement was taken from several randomly selected $10\ \mu\text{m}$ aortic sections. As is the case for any random sample, it is possible that these selected sections were not entirely representative of the aortic disease throughout the entire vessel. An alternative explanation might be that the innervation density in the unstable social group could have been underestimated due to substantial necrosis, which is characteristic of severe lesions and where innervation is absent. We have previously reported that animals in an unstable social environment exhibit more severe, advanced lesions (2).

Neo-intimal expansion during atherosclerosis is characterized by monocyte recruitment, infiltration and ultimately differentiation into tissue-resident macrophages. It has been

demonstrated that monocytes and macrophages, as well as other inflammatory cells, express and secrete NGF (42). Moreover, in an inflammatory environment, such as that in the atherosclerotic vessel wall, macrophages/monocytes overexpress NGF and NGF receptors (43). It is well established that NGF is critical for the survival, growth and development of SNS neurons (44), and inflammatory cell derived NGF has been implicated in SNS hyperinnervation in both lymph nodes (8) and the myocardium (12). In the current study, the presence of extensive SNS innervation in the atherosclerotic lesion raises the possibility that this neuronal remodeling is driven by NGF released locally by monocytes, macrophages and other inflammatory cells as the neo-intima expands.

Although the SNS has been implicated in the pathophysiology of atherosclerosis (45, 46), infectious disease (8), and cancer metastasis (47, 48), it is difficult to understand the adaptive purpose of SNS hyperinnervation in diseased tissue (i.e., why would there be such a mechanism to exacerbate disease?). Alternatively, it is likely that the initial SNS recruitment serves an adaptive, reparative function, but that over time the SNS hyperinnervation may have deleterious effects. To support this notion, it has been reported that early atherosclerotic lesions in disease-prone ApoE^(-/-) mice contained a preponderance of M2 macrophages (“alternatively activated” macrophages) that are involved in tissue repair and growth (49). In contrast, advanced lesions examined later in the disease process were characterized by M1 macrophages (“classic” macrophages) which are inflammatory. Since it has been shown that norepinephrine can stimulate monocytes/macrophages via adrenergic receptors (50, 51), it is possible that SNS activation of M2 macrophages in early lesions could aid in tissue repair, but following the phenotypic switch to M1 macrophages the same SNS activation may enhance vascular inflammation and exacerbate disease. This possibility will be examined in future work using transgenic animal models.

In summary, the current study has provided evidence that SNS innervation of the vasculature is structurally remodeled in relation to the progression of atherosclerosis, rather than social environment, in hyperlipidemic rabbits prone to the development of disease. Extensive SNS varicosities were observed within the neo-intima in animals with atherosclerotic lesions, and in the vascular media in both diseased and non-diseased animals, which is an innervation pattern not previously reported. SNS innervation patterns observed in atherosclerotic human and mouse aortas were consistent with the rabbit, suggesting that SNS hyperinnervation of the diseased vessel wall is a general feature across mammalian species. This SNS structural remodeling may be an important mechanism by which autonomic plasticity modulates atherosclerotic disease processes in the vessel wall.

Acknowledgments

This work was supported by the National Heart, Lung and Blood Institute (HL116387 to P.M.M and HL04726 to N.S.).

Abbreviations

SNS	Sympathetic Nervous System
WHHL	Watanabe Heritable Hyperlipidemic Rabbit

NZW	New Zealand White Rabbit
CHD	Coronary Heart Disease
NGF	Nerve Growth Factor
SIV	Simian Immunodeficiency Virus
TH	Tyrosine Hydroxylase
ROI	Region of Interest
ANOVA	Analysis of Variance

References

1. Kaplan JR, Manuck SB, Clarkson TB, Lusso FM, Taub DM. Social status, environment, and atherosclerosis in cynomolgus monkeys. *Arteriosclerosis (Dallas, Tex)*. 1982; 2:359–68.
2. McCabe PM, Gonzales JA, Zaias J, Szeto A, Kumar M, Herron AJ, Schneiderman N. Social environment influences the progression of atherosclerosis in the watanabe heritable hyperlipidemic rabbit. *Circulation*. 2002; 105:354–9. [PubMed: 11804992]
3. Noller CM, Szeto A, Mendez AJ, Llabre MM, Gonzales JA, Rossetti MA, Schneiderman N, McCabe PM. The influence of social environment on endocrine, cardiovascular and tissue responses in the rabbit. *International journal of psychophysiology : official journal of the International Organization of Psychophysiology*. 2013; 88:282–8. [PubMed: 22546665]
4. Paredes J, Szeto A, Levine JE, Zaias J, Gonzales JA, Mendez AJ, Llabre MM, Schneiderman N, McCabe PM. Social experience influences hypothalamic oxytocin in the WHHL rabbit. *Psychoneuroendocrinology*. 2006; 31:1062–75. [PubMed: 16963189]
5. Manuck SB, Kaplan JR, Clarkson TB. Behaviorally induced heart rate reactivity and atherosclerosis in cynomolgus monkeys. *Psychosomatic medicine*. 1983; 45:95–108. [PubMed: 6683414]
6. Kaplan JR, Manuck SB. Antiatherogenic effects of beta-adrenergic blocking agents: theoretical, experimental, and epidemiologic considerations. *American heart journal*. 1994; 128:1316–28. [PubMed: 7977013]
7. Sloan EK, Capitanio JP, Cole SW. Stress-induced remodeling of lymphoid innervation. *Brain, behavior, and immunity*. 2008; 22:15–21.
8. Sloan EK, Capitanio JP, Tarara RP, Mendoza SP, Mason WA, Cole SW. Social stress enhances sympathetic innervation of primate lymph nodes: mechanisms and implications for viral pathogenesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2007; 27:8857–65. [PubMed: 17699667]
9. Sloan EK, Nguyen CT, Cox BF, Tarara RP, Capitanio JP, Cole SW. SIV infection decreases sympathetic innervation of primate lymph nodes: the role of neurotrophins. *Brain, behavior, and immunity*. 2008; 22:185–94.
10. Sloan EK, Tarara RP, Capitanio JP, Cole SW. Enhanced replication of simian immunodeficiency virus adjacent to catecholaminergic varicosities in primate lymph nodes. *Journal of virology*. 2006; 80:4326–35. [PubMed: 16611891]
11. Glebova NO, Ginty DD. Heterogeneous requirement of NGF for sympathetic target innervation in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2004; 24:743–51. [PubMed: 14736860]
12. Hasan W, Jama A, Donohue T, Wernli G, Onyszchuk G, Al-Hafez B, Bilgen M, Smith PG. Sympathetic hyperinnervation and inflammatory cell NGF synthesis following myocardial infarction in rats. *Brain research*. 2006; 1124:142–54. [PubMed: 17084822]
13. Wernli G, Hasan W, Bhattacharjee A, van Rooijen N, Smith PG. Macrophage depletion suppresses sympathetic hyperinnervation following myocardial infarction. *Basic research in cardiology*. 2009; 104:681–93. [PubMed: 19437062]

14. Atkinson JB, Swift LL, Virmani R. Watanabe heritable hyperlipidemic rabbits. Familial hypercholesterolemia. The American journal of pathology. 1992; 140:749–53. [PubMed: 1546748]
15. Buja LM, Kita T, Goldstein JL, Watanabe Y, Brown MS. Cellular pathology of progressive atherosclerosis in the WHHL rabbit. An animal model of familial hypercholesterolemia. Arteriosclerosis (Dallas, Tex). 1983; 3:87–101.
16. Farabolini F. Behavioral and endocrine aspects of dominance and submission in male rabbits. Aggressive Behavior. 1987; 13:247–58.
17. Muscettola M, Girolami L, Tanganelli C, Fontani G, Lupo C. Immune and endocrine aspects of social and territorial behavior in male rabbits. Neuroimmunomodulation. 1995; 2:155–60. [PubMed: 8646565]
18. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A Definition of Advanced Types of Atherosclerotic Lesions and a Histological Classification of Atherosclerosis: A Report From the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation. 1995; 92:1355–74. [PubMed: 7648691]
19. Hsieh WT, Chiang BH. A well-refined in vitro model derived from human embryonic stem cell for screening phytochemicals with midbrain dopaminergic differentiation-boosting potential for improving Parkinson's disease. Journal of agricultural and food chemistry. 2014; 62:6326–36. [PubMed: 24933592]
20. Mutoh J, Ohsawa M, Hisa H. Involvement of renal sympathetic nerve activation on the progression of ischemic acute kidney injury in the mouse. Journal of pharmacological sciences. 2014; 125:415–21. [PubMed: 25069611]
21. Rodriguez-Diaz R, Abdulreda Midhat H, Formoso Alexander L, Gans I, Ricordi C, Berggren P-O, Caicedo A. Innervation Patterns of Autonomic Axons in the Human Endocrine Pancreas. Cell Metabolism. 2011; 14:45–54. [PubMed: 21723503]
22. De Camilli P, Harris SM Jr, Huttner WB, Greengard P. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. The Journal of cell biology. 1983; 96:1355–73. [PubMed: 6404911]
23. Greengard P, Valtorta F, Czernik A, Benfenati F. Synaptic vesicle phosphoproteins and regulation of synaptic function. Science. 1993; 259:780–5. [PubMed: 8430330]
24. Rosahl TW, Spillane D, Missler M, Herz J, Selig DK, Wolff JR, Hammer RE, Malenka RC, Sudhof TC. Essential functions of synapsins I and II in synaptic vesicle regulation. Nature. 1995; 375:488–93. [PubMed: 7777057]
25. Bergquist J, Tarkowski A, Ekman R, Ewing A. Discovery of endogenous catecholamines in lymphocytes and evidence for catecholamine regulation of lymphocyte function via an autocrine loop. Proceedings of the National Academy of Sciences of the United States of America. 1994; 91:12912–6. [PubMed: 7809145]
26. Mouton, PR. An Introduction for Bioscientists. John Hopkins University Press; Baltimore: 2002. Principles and Practices of Unbiased Stereology.
27. Shi SR, Chaiwun B, Young L, Cote RJ, Taylor CR. Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 1993; 41:1599–604. [PubMed: 7691930]
28. Ehrich JM, Messinger DI, Knakal CR, Kuhar JR, Schattauer SS, Bruchas MR, Zweifel LS, Kieffer BL, Phillips PE, Chavkin C. Kappa Opioid Receptor-Induced Aversion Requires p38 MAPK Activation in VTA Dopamine Neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2015; 35:12917–31. [PubMed: 26377476]
29. Neal KB, Parry LJ, Bornstein JC. Strain-specific genetics, anatomy and function of enteric neural serotonergic pathways in inbred mice. The Journal of physiology. 2009; 587:567–86. [PubMed: 19064621]
30. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona

- A. Fiji: an open-source platform for biological-image analysis. *Nature methods*. 2012; 9:676–82. [PubMed: 22743772]
31. Bennett MR, Farnell L, Gibson WG, Blair D. A quantitative description of the diffusion of noradrenaline in the media of blood vessels following its release from sympathetic varicosities. *Journal of theoretical biology*. 2004; 226:359–72. [PubMed: 14643650]
 32. Pannese, E. *Neurocytology: Fine structure of neurons, nerve processes and neuroglial cells*. 2. Switzerland: Springer; 2015. *Intercellular Junctions Involving Neurons*; p. 99-137.
 33. Gabella, G. Fine structure of post-ganglionic nerve fibres and autonomic neuroeffector junctions. In: Burnstock, G.; Hoyle, CHV., editors. *Autonomic neuroeffector mechanisms*. Chur, Switzerland: Harwood Academic; 1992. p. 1-31.
 34. Luff SE. Ultrastructure of sympathetic axons and their structural relationship with vascular smooth muscle. *Anatomy and embryology*. 1996; 193:515–31. [PubMed: 8737808]
 35. Libby P, Hansson GK. Inflammation and Immunity in Diseases of the Arterial Tree: Players and Layers. *Circulation research*. 2015; 116:307–11. [PubMed: 25593275]
 36. Cavallotti C, Bruzzone P, Mancone M. Catecholaminergic nerve fibers and beta-adrenergic receptors in the human heart and coronary vessels. *Heart and vessels*. 2002; 17:30–5. [PubMed: 12434199]
 37. Kienecker EW, Knoche H. Sympathetic innervation of the pulmonary artery, ascending aorta, and coronar glomera of the rabbit. A fluorescence microscopic study. *Cell and tissue research*. 1978; 188:329–33. [PubMed: 647754]
 38. Stanojevic S, Dimitrijevic M, Kustrimovic N, Mitic K, Vujic V, Leposavic G. Adrenal hormone deprivation affects macrophage catecholamine metabolism and beta2-adrenoceptor density, but not propranolol stimulation of tumour necrosis factor-alpha production. *Experimental physiology*. 2013; 98:665–78. [PubMed: 23180813]
 39. Bhangu PS, Genever PG, Spencer GJ, Grewal TS, Skerry TM. Evidence for targeted vesicular glutamate exocytosis in osteoblasts. *Bone*. 2001; 29:16–23. [PubMed: 11472886]
 40. Matsumoto K, Ebihara K, Yamamoto H, Tabuchi H, Fukunaga K, Yasunami M, Ohkubo H, Shichiri M, Miyamoto E. Cloning from insulinoma cells of synapsin I associated with insulin secretory granules. *The Journal of biological chemistry*. 1999; 274:2053–9. [PubMed: 9890964]
 41. Bustos R, Kolen ER, Braiterman L, Baines AJ, Gorelick FS, Hubbard AL. Synapsin I is expressed in epithelial cells: localization to a unique trans-Golgi compartment. *Journal of cell science*. 2001; 114:3695–704. [PubMed: 11707521]
 42. Frossard N, Freund V, Advenier C. Nerve growth factor and its receptors in asthma and inflammation. *European journal of pharmacology*. 2004; 500:453–65. [PubMed: 15464052]
 43. Caroleo MC, Costa N, Bracci-Laudiero L, Aloe L. Human monocyte/macrophages activate by exposure to LPS overexpress NGF and NGF receptors. *Journal of neuroimmunology*. 2001; 113:193–201. [PubMed: 11164902]
 44. Levi-Montalcini R. The nerve growth factor 35 years later. *Science*. 1987; 237:1154–62. [PubMed: 3306916]
 45. Kaplan JR, Manuck SB, Adams MR, Weingand KW, Clarkson TB. Inhibition of coronary atherosclerosis by propranolol in behaviorally predisposed monkeys fed an atherogenic diet. *Circulation*. 1987; 76:1364–72. [PubMed: 3677359]
 46. Manuck SB, Marsland AL, Kaplan JR, Williams JK. The pathogenicity of behavior and its neuroendocrine mediation: an example from coronary artery disease. *Psychosomatic medicine*. 1995; 57:275–83. [PubMed: 7652128]
 47. Lamkin DM, Sloan EK, Patel AJ, Chiang BS, Pimentel MA, Ma JC, Arevalo JM, Morizono K, Cole SW. Chronic stress enhances progression of acute lymphoblastic leukemia via beta-adrenergic signaling. *Brain, behavior, and immunity*. 2012; 26:635–41.
 48. Sloan EK, Priceman SJ, Cox BF, Yu S, Pimentel MA, Tangkanangnukul V, Arevalo JM, Morizono K, Karanikolas BD, Wu L, Sood AK, Cole SW. The sympathetic nervous system induces a metastatic switch in primary breast cancer. *Cancer research*. 2010; 70:7042–52. [PubMed: 20823155]

49. Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, Clement M, Dussiot M, Levillain O, Graff-Dubois S, Nicoletti A, Caligiuri G. Macrophage plasticity in experimental atherosclerosis. *PloS one*. 2010; 5:e8852. [PubMed: 20111605]
50. Bierhaus A, Wolf J, Andrassy M, Rohleder N, Humpert PM, Petrov D, Ferstl R, von Eynatten M, Wendt T, Rudofsky G, Joswig M, Morcos M, Schwaninger M, McEwen B, Kirschbaum C, Nawroth PP. A mechanism converting psychosocial stress into mononuclear cell activation. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:1920–5. [PubMed: 12578963]
51. Flierl MA, Rittirsch D, Nadeau BA, Sarma JV, Day DE, Lentsch AB, Huber-Lang MS, Ward PA. Upregulation of phagocyte-derived catecholamines augments the acute inflammatory response. *PloS one*. 2009; 4:e4414. [PubMed: 19212441]

Figure 1. Identification of intima and media in rabbit aortic sections. Tissues were stained with DAPI to identify areas of cellularity for a NZW (A) or WHHL (B) rabbits. Delineation of Media and Intima or Neo-intima, outlined in white, was identified by the border of the inter-elastic lamina.

Figure 2. Identification of aortic sympathetic varicosities in WHHL rabbit. (A) Synapsin immunofluorescence shown in red, (B) TH shown in green, and (C) merge of A and B showing colocalization (seen as yellow), indicative of sympathetic varicosities. Panels D, E and F show primary antibody isotype controls for panels A, B and C, respectively. Confocal images were obtained with a 40X objective lens.

Figure 3.

Representative 40X confocal images (selected from 24 NZWs and 22 WHHLs; 3 slides per animal) showing SNS varicosities in rabbit aortic arch. (A, C) NZW and WHHL aortas, respectively, showing co-localization of TH and Synapsin (yellow), (B, D) enlargement of insets from panels A and C that includes nuclear stain (DAPI, blue). White line in panel C and D indicate border between vascular media and atherosclerotic lesion (neointima), whereas in panels A and B there is no disease (neointima) in the NZWs. Note dense SNS varicosities in the vascular media in both rabbit strains, and extensive SNS innervation of the neointima in the WHHL.

Figure 4.

Sympathetic innervation density in the thoracic aortic and aortic arch of NZW (n =24) and WHHL (n = 22) rabbits. Box plot (highlighting 5th, 25th, median, 75th, and 95th percentile of the data) of innervation density was measured in cross sectional aortic sections from each rabbit strain, and number of varicosities were expressed as counts/total aortic area (mm²). There was greater innervation density in WHHLs relative to NZWs in the aortic arch and thoracic aorta (both comparisons, $p < 0.01$).

Figure 5.

The relationship between the number of SNS varicosities and the intimal area of the aorta. (A, B) In the WHHLs, the number of intimal SNS varicosities increased as neointimal area expanded in the aortic arch ($r(21) = .855$, $p < .001$) and thoracic aorta ($r(22) = .789$, $p < .001$) due to atherosclerotic disease progression. (C, D) There was no relationship between the number of intimal varicosities and the intimal area for the NZWs that had virtually no disease.

Figure 6.

Confocal immunohistochemical and light microscopic images of a representative WHHL aorta (selected from 22 WHHLs; 3 slides per animal). (A) 10X light microscopic image of H & E stained aorta in WHHL with extensive atherosclerosis (same animal as panels B-F), (B) 20X tiled image of tyrosine hydroxylase (TH, green) labeling (suggesting numerous SNS varicosities throughout the vessel, including the neointima), (C, D, E) 40X images of the area in the highlighted box in panel B showing TH (white), macrophages (RAM-11, red), and Nerve Growth Factor (NGF, green), respectively, (F) Merged 40X image showing SNS varicosities (TH, white), macrophages (RAM-11, red) and Nerve Growth Factor (NGF, green). In addition, nuclei were stained with DAPI (blue). The co-localization of macrophages and NGF appears as yellow. Note the co-localization of NGF and macrophages proximal to the TH-labeling, as well as the absence of TH-labeling within regions of necrosis (seen as dark areas devoid of staining).

Figure 7.

SNS varicosities in mouse aortic arch (selected from 4 ApoE and 4 wildtype mice). (A, C) H & E stained wildtype and ApoE^(-/-) mouse aortas, respectively. Note extensive atherosclerosis in the ApoE^(-/-) aorta. (B, D) 40X confocal images of sections nearby the sections in panels A & C showing TH (green) and Synapsin (red) with co-localization appearing yellow in wildtype and ApoE^(-/-) aortas, respectively. Note SNS varicosities in the media of both diseased and non-diseased aortas, and extensive varicosities in the atherosclerotic neointima of the ApoE^(-/-) aorta.

Figure 8.

SNS varicosities in a human aorta (selected from 4 human aortas) with evident atherosclerosis. 40X confocal image showing TH (green) and Synapsin (red) with co-localization appearing yellow. White line delineates the intimal-medial border. Note dense innervation in both the media and atherosclerotic neointima.

Table 1Percent Time in Behavior by Group (Mean $\pm$ SEM)

	WHHL		NZW		COMBINED	
	Mean	SEM	Mean	SEM	Mean	SEM
<i>Agonistic</i>						
Unstable	42.7	± 3.2	44.6	± 4.3	43.7	± 2.7
Stable	23.9	± 3.6	29.6	± 5.1	26.8	$\pm 3.1^*$
Group Average	33.3	± 3.0	37.1	± 2.9		
<i>Affiliative</i>						
Unstable	14.6	± 1.9	16.0	± 1.8	15.3	± 1.3
Stable	24.1	± 2.1	27.6	± 2.2	25.8	$\pm 1.5^*$
Group Average	19.4	± 1.4	21.8	± 1.4		
<i>Other Non-Agonistic</i>						
Unstable	18.0	± 2.1	15.7	± 1.8	16.8	± 1.4
Stable	29.9	± 2.3	21.5	± 2.1	25.7	$\pm 1.6^*$
Group Average	24.0	± 1.5	18.6	$\pm 1.4^{\dagger}$		
<i>Inactivity</i>						
Unstable	24.7	± 1.7	23.9	± 1.5	24.3	± 1.1
Stable	22.4	± 1.8	21.8	± 1.8	22.1	± 1.3
Group Average	23.5	± 1.2	22.8	± 1.2		

* $p < 0.001$ between Unstable vs. Stable for the combined rabbit strains by ANOVA † $p = 0.013$ between WHHL and NZW mean comparisons by ANOVA

WHHLs: Unstable (N = 12), Stable (N = 10)

NZWs: Unstable (N = 14), Stable (N = 10)

Combined Rabbit Strains: Unstable (N = 26), Stable (N = 20)

Table 2

Aortic SNS innervation density (varicosities/mm²) as a function of social environment (mean ± SEM).

		NZW	WHHL
Aortic Arch	Stable	1347 ± 110	1535 ± 60
	Unstable	1136 ± 56	1540 ± 102
Thoracic Aorta	Stable	896 ± 54	1519 ± 116
	Unstable	816 ± 57	1435 ± 114

No significant differences between stable and unstable social environments in either aortic region in NZW and WHHL rabbits by ANOVA, NZW: Aortic Arch, $p = 0.078$, Thoracic Aorta, $p = 0.34$; WHHL: Aortic Arch, $p = 0.97$, Thoracic Aorta, $p = 0.61$.

For WHHLs: N=12 for Unstable and N=10 for Stable

For NZWs: N=14 for Unstable and N=10 for Stable