EFFECT OF HIGH-FAT DIET ON SYMPATHETIC NEUROTRANSMISSION IN MESENTERIC VASCULATURE IN DAHL SALT-SENSITIVE HYPERTENSION

By

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ABSTRACT

EFFECT OF HIGH-FAT DIET ON SYMPATHETIC NEUROTRANSMISSION IN MESENTERIC VASCULATURE IN DAHL SALT-SENSITIVE HYPERTENSION

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Obesity is a risk factor for cardiovascular and metabolic disorders. The prevalence of obesity-associated hypertension has been rising globally, and it is mainly linked to the increasing consumption of processed as well as fast foods. The current trend indicates that women are more likely to become obese than men; however, the onset of hypertension is delayed in obese women until they reach postmenopausal age. Several reasons have been given for such disparity, such as reproductive hormones and distribution of body fat. In addition, obesity-induced hypertension is indicated in the overdrive of sympathetic nerves centrally and peripherally. For example, sympathetic denervation in the kidneys and celiac ganglionic blockade have demonstrated the role of sympathetic nerves in hypertension. Nevertheless, there is little knowledge regards to the effect of obesity on the underlying mechanisms in the sympathetic neurotransmission in the mesenteric blood vessels. Therefore, we tested the hypothesis that high fat diet (HFD) compared to control diet (CD) results in greater sympathetic neurotransmission and nerve distribution in the Dahl salt-sensitive (Dahl ss) rat. HFD fed rats gained more body weight than rats on CD, and males became more obese than females. Mean arterial pressure (MAP) was greater in HFD versus CD at 17- and 24-wk in both sexes. Nevertheless, males and females became equally hypertensive on both diets. In mesenteric artery (MA), neurogenic constriction was higher in HFD versus CD at 17-wk in males; however, this observation was not supported by changes in vascular

reactivity or nerve density at the same time point. ATP is co-released with norepinephrine (NE) from the presynaptic nerves and it mediates purinergic neurotransmission. Moreover, the purinergic nerves were low in count at this time point indicating minimal contribution to the higher neurogenic response in HFD at 17-wk in males. In mesenteric vein (MV), neurogenic response was greater in HFD versus CD at 17-wk in males (similar to MA). There was also a greater adrenergic venous reactivity (norepinephrine mediated) and higher tyrosine hydroxylase (TH; sympathetic nerve marker) nerve density in HFD versus CD at 17-wk in males. This suggests HFD-induced hypertension is partly driven by adrenergic nerves from MV at 17-wk in males. Finally, HFD increased in most cases the sympathetic vesicles in the nerve cell bodies in celiac ganglion (CG), and sympathetic nerve fibers in MA and MV. In addition, three distinct populations of sympathetic vesicles/nerves were identified in CG, MA, and MV, namely TH-immunoreactive (TH-ir), vesicular nucleotide transporter (VNUT-ir; ATP marker), and TH/VNUT colocalization. The distribution of adrenergic, purinergic, and colocalized vesicles in cell bodies at the CG was not necessarily reflected in the periarterial and perivenous nerves. Taken together, HFD-associated hypertension is not driven by changes in the sympathetic neurotransmission from the mesenteric vasculature in male and female Dahl ss rats.

This thesis is dedicated to Meazaye and Dr. Hermann Gmeiner!

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

α1-AR	α1-adrenergic receptor
α2-AR	α2-adrenergic receptor
AC	Adenyl cyclase
ACE	Angiotensin-converting enzyme
ADH	Antidiuretic hormone
AFR	Africa
Aldo	Aldosterone
AMR	America
Ang II	Angiotensin II
AP	Action potential
AR	Androgen receptor
AT1	Angiotensin II receptor subtype I
ATP	Adenosine triphosphate
β-AR	β-adrenergic receptor
BKca	Calcium activated big potassium ion channel
BMI	Body mass index
Ca++	Calcium ion
cAMP	cyclic adenosine monophosphate
CCBs	Calcium channel blockers
CD	Control diet
CDC	Centers for disease control and prevention

CG	Celiac ganglion
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene-related peptide
CKD	Chronic kidney disease
CM	Calmodulin
CNS	Central nervous system
CO	Cardiac output
CO2	Carbon dioxide
CRP	C-reactive protein
DA	Dopamine
DAG	Diacylglycerol
Dahl ss	Dahl salt-sensitive
DBH	Dopamine β-hydroxylase
DBP	Diastolic blood pressure
DD	DOPA decarboxylase
DHPs	Dihydropyridines
DMSO	Dimethyl sulfoxide
DMT	Danish Myo Technology
DOCA	Deoxycorticosterone acetate
Dpi	Dots per inch
EFS	Electrical field stimulation
ENDF	Endothelium Derived hyperpolarizing factor
ERα	Estrogen receptor α

ERβ	Estrogen receptor β
ESRD	End-stage renal disease
ET-1	Endothelin-1
EUR	Europe
FFAs	Free fatty acids
GDP	Guanosine diphosphate
GPCR	G protein coupled receptor
GPR30	G protein coupled estrogen receptor (GPER)
GTP	Guanosine triphosphate
GWAS	Genome-wide association studies
H_2O	Water
HFD	High-fat diet
HPLC	High-performance liquid chromatography
Hz	Hertz
IL	Interleukin
IP_3	Inositol 1,4,5-triphosphate
Ir	Immunoreactive
K^+	Potassium ion
µm	Micrometer (micron)
µM	Micromolar
M	Molar
MA	Mesenteric artery
MAP	Mean arterial pressure

MCP-1	Monocyte chemoattractant protein-1
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
mmHg	millimeter mercury
MV	Mesenteric vein
N.A.	Numerical aperture
Na^+	Sodium ion
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NE	Norepinephrine
NET	Norepinephrine transporter
NO	Nitric oxide
NPY	Neuropeptide Y
O2	Oxygen
PB	Phosphate buffer
PGI_2	Prostacyclin
PKA	Phosphokinase A
PLC	Phospholipase C
PR	Progesterone receptor
RAAS	Renin-angiotensin-aldosterone-system
ROS	Reactive oxygen species
Rpm	Revolution per minute
RSNA	Renal sympathetic nerve activity
SBP	Systolic blood pressure

SCAT	Subcutaneous adipose tissue
SER	South east Asia region
SNPs	Single nucleotide polymorphisms
SNS	Sympathetic nervous system
SNV	Sympathetic nerve varicosity
SP	Substance P
SVR	Systemic vascular resistance
TH	Tyrosine hydroxylase
TNF-α	Tumor necrosis factor-α
TPR	Total peripheral resistance
TTX	Tetrodotoxin
TV	Television
TXA_2	Thromboxane
Tyr	Tyrosine
VAT	Visceral adipose tissue
VNUT	Vesicular nucleotide transporter
VSMC	Vascular smooth muscle cell
WHO	World health organization
Wk	week
WPR	West pacific region

CHAPTER 1: GENERAL INTRODUCTION

1.0 Obesity

1.1 Prevalence of Obesity

Obesity is defined as a body mass index (BMI) ≥ 30.0 kg/m^2 (Poirier et al., 2006b). Large waist circumference along with obesity are indicators of health risks, such as cardiovascular disease, type 2 diabetes, and hypertension (Table 1). Obesity is a global health issue with current trends indicating an increase in prevalence. According to the world health organization (WHO) statistics from 2016, close to 650 million adults (18 years and older) were obese. In addition, more than 340 million children and teenagers were either overweight or obese in 2016 (World Health Organization, 2018). A recent review (Bluher, 2019) reports that despite differences among countries, global obesity has been rising at an alarming rate. This trend is evident in all age groups from both sexes worldwide. However, there is a slightly higher increasing rate in girls and women compared to boys and men (Fig. 1) (Kanter and Caballero, 2012; WHO, 2013; Bluher, 2019). The obesity pandemic is also becoming a health concern in the developing countries (Poobalan and Aucott, 2016).

A 2017 report from the Centers for Disease Control and Prevention (CDC) indicates that the prevalence of obesity was 18.5 and 39.8 % in young and adult populations, respectively. Furthermore, obesity is more common in middle-aged than in younger adults. Similarly, obesity is widespread in the Hispanic and non-Hispanic black population in the United States (Hales et al., 2017). Like at the global level, the trend of obesity prevalence is greater in women than men in the United States (Kanter and Caballero, 2012; Ogden et al., 2012). Sex difference in the obesity prevalence trend

is slightly higher in males compared to females in children and adolescents between 2 and 19 years of age.

Table 1. Categorization of body weight by BMI, and waist circumference. Data are shown with associated health risks for cardiovascular disease, type 2 diabetes, and hypertension (Increased, high, very high, and extremely high) (Poirier et al., 2006b).

	BMI (kg/m^2)	**Men, ≤102 cm; Women, ≤88 cm**	**Men, >102 cm; Women, >88 cm**
Underweight	<18.5	---	---
Normal	18.5 – 24.9	---	---
Overweight	25.0 – 29.9	Increased	High
Obesity, class			
I	30.0 – 34.9	High	Very high
II	35.0 – 39.9	Very high	Very high
III (extreme obesity)	≥40	Extremely high	Extremely high

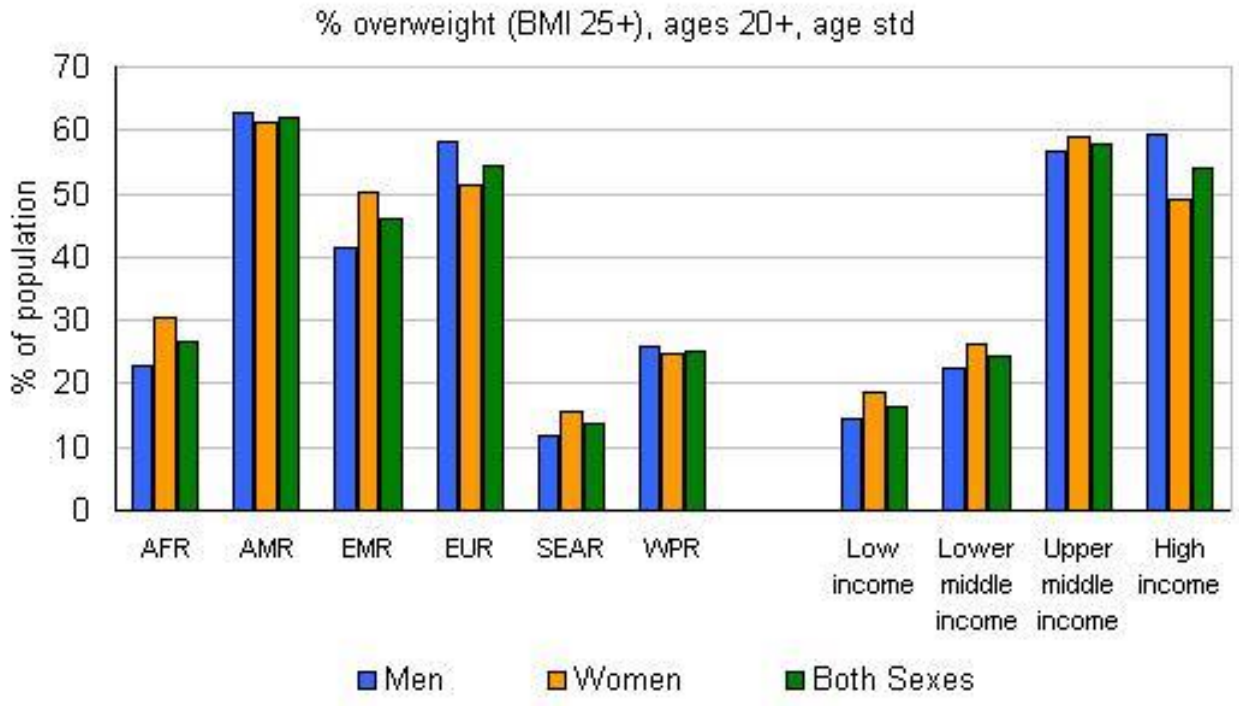


Figure 1. Global trend of obesity prevalence (in %) between men and women. **.** Data standardized to age. Data compared from Africa (AFR), America (AMR), Eastern Mediterranean, Europe (EUR), South East Asia Region (SEAR), and West Pacific Region (WPR). Hypertension is also compared by income (low, lower middle, upper middle, high). (WHO, 2013).

1.2 Factors Contributing to Obesity

Obesity is the product of an energy imbalance between intake and expenditure of calories resulting in an excess energy that is stored as a fat (Hill et al., 2012). There are social, economic, environmental, and biological factors that contribute to the development of obesity. Social behavior encompasses sedentary lifestyle, lack of exercise, lack of sleep, increased time spent on watching TVs and playing video games. When these factors are combined with the inability to afford healthy foods, it accelerates accumulation of fat. Families in the lower strata of economic level are compelled to consume highly processed foods (Poti et al., 2017), such as fast foods (Rosenheck, 2008). This lifestyle has a profound long-term consequence on children and adults. However, people who are wealthy and able to afford consuming large quantity of food could also become obese. Therefore, individuals at the lower- and upper-income levels may be at risk in becoming obese for different reasons. Lack of education is also a variable in the development of obesity. For example, individuals that are not aware of the health risks posed by certain diets may lead unhealthy life. Bad habits such as smoking and excessive alcohol consumption are also contributing factors to obesity (Hruby and Hu, 2015).

Biological factors including genetics, and metabolism play a crucial role in obesity. The genetic basis of obesity is mainly linked to heredity. Gene mutation studies have demonstrated that certain genes such as the *ob* gene (encodes leptin) is essential in regulating appetite and energy expenditure. Alteration, in any way, of this gene will have a dire consequence for maintaining normal body weight (Friedman, 2009). The metabolic efficiency varies between individuals. The net energy expenditure is negative

in obese individuals due to lower resting metabolic rate, and the inability to dispose or use thermal energy (Oussaada et al., 2019).

1.3 Fat Distribution in Obesity

Although obesity, in general, poses health risks, the location of fat accumulation is a more important indicator of health status (Frank et al., 2018). In addition, fat depots are not homogenous in composition and contribution to metabolic disorders. Adipose tissue is dynamic and is the site where leptin and adiponectin are produced (Greenberg and Obin, 2006). Leptin is mainly involved in controlling appetite whereas adiponectin regulates fatty acid metabolism and glucose level (Feijóo-Bandín et al., 2016). Moreover, adipocytes are essential in metabolic homeostasis and thermoregulation. There are several body locations where adipocytes accumulate, including the subcutaneous, visceral, bone marrow, and intermuscular. However, most fat tissue are deposited either subcutaneously or viscerally. Subcutaneous adipose tissue (SCAT) accumulates close to 80-90% of fat whereas visceral adipose tissue (VAT) holds between 6 and 20% (Frank et al., 2018). SCAT is mainly found in the abdominal, subscapular, gluteal, and femoral regions whereas VAT is found in the abdominal organs such as the liver and intestines (Ibrahim, 2010).

The total body fat weight is generally higher in women than men. Despite that women have lower cardiovascular risk associated with obesity. That is because most of the adipose tissue is accumulated in the hips and thighs (gluteal and femoral) in obese women forming a gynoid obesity (“pear” shaped) whereas fat is primarily deposited in the abdominal region in obese men creating and android obesity (“apple” shaped) (Wiklund et al., 2008; Manolopoulos et al., 2010; Karastergiou et al., 2012) (Fig. 2).

Nevertheless, the adipocytes in the lower body in women are believed to provide some protection from metabolic disorders such as atherosclerosis and type 2 diabetes (Karastergiou et al., 2012). In addition, SCAT drains to the venous system whereas the VAT drains to the liver. In doing so, the FFAs released from VAT evoke gluconeogenesis leading to lipolysis, hyperinsulinemia, dyslipidemia, and insulin resistance (Karastergiou and Fried, 2013; Feijóo-Bandín et al., 2016).

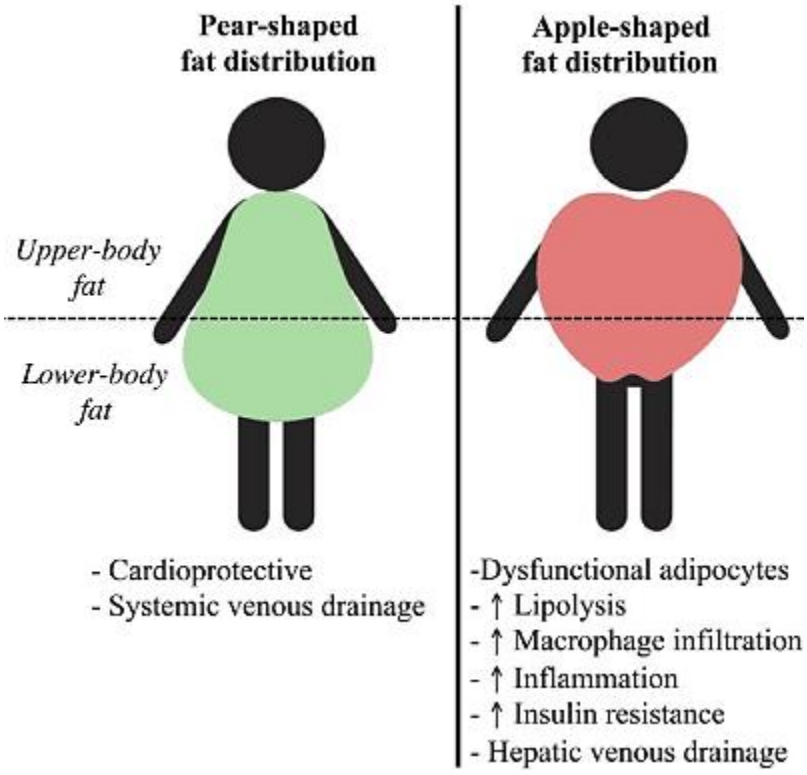


Figure 2. Fat distribution in men and women. Most of subcutaneous fat accumulated in the lower part of the body (thighs and hips; gynoid) decreasing cardiovascular risk. In men, the accumulation of abdominal (visceral fat) makes them vulnerable to health conditions, such as inflammation, and insulin resistance (Feijóo-Bandín et al., 2016).

1.4 Mechanisms Involved in Obesity-related Diseases

Obesity is a risk factor for cardiovascular disease, stroke, type 2 diabetes, hypertension, cancer, dyslipidemia, atherosclerosis, and liver disease (Kyrou et al., 2000). Obesity influences the cardiovascular system in several ways. Obesity itself can cause cardiovascular problems. For instance, the demand for blood perfusing to the increased adipose tissue overworks the heart. This compels the heart to increase its stroke volume and cardiac output that eventually leads to left ventricle hypertrophy and diastolic dysfunction (Summers et al., 1996; Mathew et al., 2008). Fat in the heart could also interfere with the heart's electrical conduction system and block sinoatrial, bundle branch, and atrioventricular signals (Balsaver et al., 1967). The adipocytes may also secret adipokines that injure myocardial cells. Finally, accumulation of fat may induce lipotoxicity to the myocardial cells (Zhou et al., 2000). Congestive heart failure (Poirier et al., 2006a), arrythmias (Kannel et al., 1988), and coronary artery disease (Hubert et al., 1983) are also related to obesity.

Narrowing of arteries by plaque (atherosclerosis) in the brain leads to ischemic stroke (Kroll et al., 2016). Hypertension induced by obesity could also cause hemorrhagic stroke(Kroll et al., 2016). In obesity-linked dyslipidemia, the levels of triglycerides, low-density lipoprotein, apolipoprotein B, and free fatty acids are increased while high-density lipoprotein decreases (Klop et al., 2013). Type 2 diabetes accompanied by insulin resistance is associated with obesity because of lipotoxicity of β-cells in the pancreas (Cerf, 2013; Al-Goblan et al., 2014). Additionally, obesity is linked with cancer by the release of inflammatory and anti-tumor suppressor factors

from adipocytes (Stone et al., 2018). Finally, obesity is known to induce liver damage due to imbalance between fatty acid uptake and oxidation (Fabbrini et al., 2010).

1.5 Socio-economic Implications of Obesity

Childhood obesity has a long-term consequence that, unless controlled, could affect quality of life in adulthood. Lack of exercise, and exposure to unhealthy diets are the main contributing factors to childhood obesity. Children and adults who are obese will have cardiovascular and other co-morbidities. Moreover, obesity affects the emotional, and social welfare of individuals (Russell-Mayhew et al., 2012). Obese people are socially stigmatized that affects their self-esteem. There is also an economic burden on families that deal with an obese member (Enzi, 1994). Overall, obesity can be a burden to the obese individual, to his or her family, and to society (Trasande and Chatterjee, 2009).

1.6 Current Interventions to Control Body Weight

The current approaches for managing obesity can be mainly divided into four categories. First, change in lifestyle by avoiding high-calorie, and processed foods, and increase physical activity. However, cost of and access to healthy food presents a challenge. Additionally, lack of patient compliance to lifestyle changes limit progress. Second, using drugs for weight loss such as Xenical, Qsymia, Belviq, Contrave, and Saxenda is a clinical approach for the treatment of prolonged obesity. Absence of insurance coverage and a properly designed public policy hinder achieving the intended outcome. Third, bariatric surgery for severe obesity, although it does not yield the anticipated result for every patient. This ineffectiveness is attributed to impaired hypothalamic neurons in these individuals. Fourth, medical devices such as gastric

bands that limit food intake, electrical stimulation of the vagus nerve to inhibit food intake, space-occupying gastric balloons, and gastric emptying and drainage using a tube attached to the stomach prior to absorption (Heymsfield et al., 2018).

2.0 Hypertension

2.1 Homeostasis of Blood Pressure

Blood pressure is defined as the force of blood exerted on the walls of arteries with each heartbeat. Blood pressure is often reported as systolic over diastolic pressure. Systolic blood pressure (SBP) is the maximum pressure when the ventricles contract to pump blood out of the heart. Diastolic blood pressure (DBP) is the minimum pressure recorded when the ventricles relax and fill with blood before the following contraction. The normal level of arterial blood pressure is 120/80 mmHg. Blood pressure is also reported as mean arterial pressure (MAP), which is the average blood pressure from a single cardiac cycle. This can be calculated from SBP and DBP (MAP = DBP+1/3 (SBP-DBP)). MAP is also the product of cardiac output (CO) and total peripheral resistance (TPR) (Brzezinski, 1990).

Blood pressure allows blood to perfuse tissues throughout the body in order to deliver nutrients, and oxygen as well as to remove waste from tissues. Maintaining a normal blood pressure is essential for a healthy heart, blood vessels, and kidney. The kidney and the brain are the two organs that mainly control blood pressure (Nishi et al., 2015). When blood pressure increases, the kidney removes water and salt from the blood to the urine. This in turn decreases blood volume, CO and blood pressure. The opposite process would happen if blood pressure drops. The renin-angiotensin system plays important role for the kidney to control blood volume (Guyton, 1991). On the other hand, the brain controls blood pressure through the baroreflex system integrated into the medulla oblongata for a short-term control (Colombari et al., 2001). If blood pressure increases, baroreceptors in the carotid sinus and in the aortic arch activate the

parasympathetic nerves to lower heart rate and increase arterial vasodilation. Sympathetic nerves also regulate blood pressure that will be discussed in part 4.0 of chapter 1.

Table 2. Classification of blood pressure (Whelton et al., 2018; Muntner et al., 2019).

Blood pressure	**Systolic, mmHg (top number)**		**Diastolic, mmHg (bottom number)**
Normal	<120	and	<80
Elevated	120 – 129	and	<80
Hypertension stage 1	130 – 139	or	80 – 89
Hypertension stage 2	>140	or	>90
Hypertension crisis	>180	and/or	>120

2.2 Prevalence of Hypertension

Hypertension is a clinical term for high blood pressure at rest with greater than 130/80 mmHg systolic/diastolic measurements (Carey et al., 2018). The prevalence of hypertension has been increasing at an alarming rate globally. Data collected between 2000 and 2010 shows close to 1.39 billion people, and 31.1% of adult (older than 20 years) population were hypertensive (Bloch, 2016). Hypertension trend is slightly higher in men than women worldwide; however, the overall trend in both sexes indicate a decrease in the developed world, and increase the less developed regions of the world (WHO, 2013; Mills et al., 2016) (Fig. 3). In the United States (US), there are more than 75 million adults (older than 18 years) with hypertension (Merai et al., 2016). Hypertension incidence is more prevalent in men than women across all races in the US. However, this sex difference disappears after age 60 (Sandberg and Ji, 2012; Gillis and Sullivan, 2016).

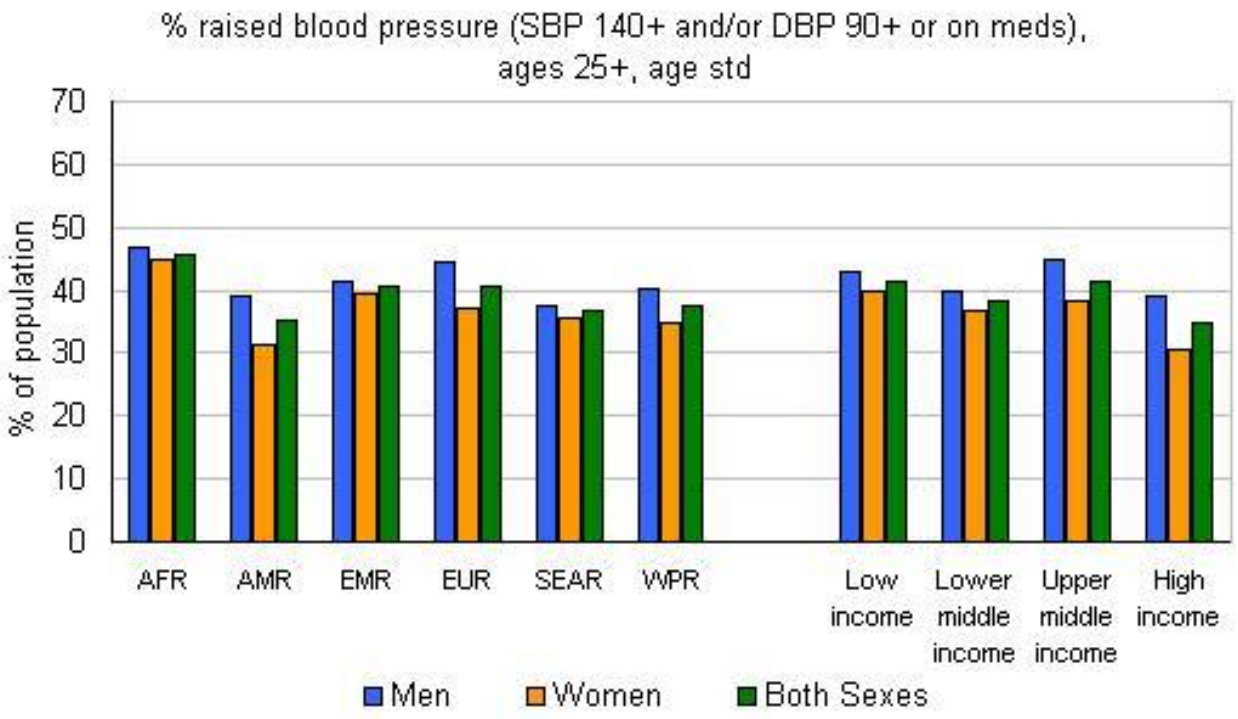


Figure 3. Global prevalence of hypertension compared by sex, and national income. Data standardized to age. Data compared from Africa (AFR), America (AMR), Eastern Mediterranean, Europe (EUR), South East Asia Region (SEAR), and West Pacific Region (WPR). Hypertension is also compared by income (low, lower middle, upper middle, high). (WHO, 2013).

2.3 Types of Hypertension

There are three types of hypertension: primary (essential), secondary, and resistant. Primary hypertension has no known cause, and it accounts for nearly 95% of all hypertension cases (Carretero and Oparil, 2000). This is the type of hypertension discussed in this dissertation unless otherwise specified. On the other hand, secondary hypertension has known causes and it is common in about 10% of adults. Some of the common causes of secondary hypertension are Cushing's Syndrome, obstructive sleep apnea, renal parenchymal disease, renal artery stenosis, and primary aldosteronism (Rimoldi et al., 2014). Resistant hypertension is the type of hypertension that does not respond to treatments and may require combining several drugs to control blood pressure. Secondary hypertension and in some cases primary hypertension are considered resistant hypertension. Nevertheless, resistant hypertension is often thought to be caused by untreated secondary hypertension (Yaxley and Thambar, 2015).

2.4 Factors Attributed to Hypertension

There is no compelling evidence to suggest direct genetic basis for hypertension although genome-wide association (GWAS) studies have narrowed this missing link by indicating variation in 13 single nucleotide polymorphisms (SNPs) in SBP and 20 for DBP (Butler, 2010). Most of these genes are related to enzymes, ion channels and receptors involved in RAAS (salt-water balance and blood volume), hormonal control of blood pressure, and vascular receptors. Despite the generally accepted notion that primary hypertension is idiopathic, there are some factors (other than genetic) that are associated with hypertension. These include obesity, high alcohol consumption,

cigarette smoking, insulin resistant, high salt intake, ageing, stress, low potassium and calcium levels, and lack of exercise (Carretero and Oparil, 2000).

2.5 Relationship between Hypertension and Cardiovascular Disease

The heart is a vital organ involved in pumping oxygenated blood throughout the body via arteries. Chronic hypertension increases cardiovascular risks by inflicting injury to the heart and blood vessels in the following ways (Oparil et al., 2003). High blood pressure can damage the coronary artery leading to narrow or obstructed blood vessel. This obstruction leads to arrhythmias (irregular heartbeats), chest pain, and heart attack (Escobar, 2002). Chronic exposure of the heart to hypertension can also increase the workload that the heart has to undertake. Consequently, the left ventricle enlarges (hypertrophy) and weakens to the point which it cannot sufficiently pump blood to the rest of the body. This, in turn, results in heart failure, heart attack, and cardiac arrest. In addition, long-term hypertension fatigues the cardiac muscle compromising its function (Aronow, 2017; Shenasa and Shenasa, 2017). The assault on the heart due to hypertension may eventually kill the person. Hypertension can damage the lumen of peripheral arteries. Subsequently, plaque may build up on the damaged sites further recruiting inflammatory factors, and then narrowing the lumen restricting blood flow (atherosclerosis) (Lithell, 1994). Unfortunately, this localized damage will force the heart to work harder to push blood through the clot. Moreover, the artery may lose its elastic ability to accommodate for larger volume of blood flow (Safar et al., 2018).

Chronic high blood pressure can negatively impact the function of cerebral arteries. The end result can be a stroke leading to death or lifelong disability. Hypertension can elicit two types of stroke: ischemic stroke and hemorrhagic stroke.

Most often clots from other regions of the body would block the passage of oxygenated blood to brain cells. If this situation is not reversed quickly, neurons may die (Sharma, 2016). In the case of hemorrhagic stroke, the prolonged high pressure weakens a spot in the artery forming a bulge known as aneurysm. Aneurysm can also occur in other parts of the body, such as in aorta and abdominal blood vessels. When the aneurysm ruptures, it causes an internal bleeding and stroke (Ko and Yoon, 2017). This can be fatal since it may be too late when the symptoms are diagnosed.

Cardiovascular disease manifests differently in children compared with adults suggesting that cardiovascular events are influenced by age. For instance, heart attack, death, and stroke are common in adults whereas vascular damage and left ventricular hypertrophy are commonly observed in children (Drozdz and Kawecka-Jaszcz, 2014). Age-dependent sex differences in cardiovascular disease are well established. Men succumb to cardiovascular disease such as coronary heart disease at younger age than women. Conversely, older women suffer from stroke compared to age-matched men. Despite improved treatments for cardiovascular disease, this trend continues (Bots et al., 2017).

2.6 Links between Hypertension and Diabetes

Hypertension is associated with more than 50% of type 2 diabetes mellitus (diabetes) cases. Diabetes on its own accelerates cardiovascular disease such as stroke, cardiac arrest, arrhythmia, and coronary artery disease. The risk for cardiovascular disease or even mortality increases exponentially if hypertensive individuals are diabetic as well (Chen et al., 2011; Lastra et al., 2014). Hypertension is more prevalent in men than women before menopause (Chen et al., 2011). However,

some studies have reported that diabetes is less prevalent in men compared to women (Hu and Group, 2003). The exact mechanism for sex difference in diabetes is not sufficiently established.

Hypertension and diabetes have common risk factors such as obesity, sedentary lifestyle, and unhealthy dieting habit. For these and other reasons they co-occur together. It is also known that one can cause the other. Interestingly, they also share common disease mechanisms including insulin resistance, obesity, inflammation, and oxidative stress (Stump et al., 2005; Cheung and Li, 2012). Insulin is a hormone produced in the β-cells in the pancreas, which plays a crucial role in the homeostasis of glucose. Its main function is to facilitate the transport of glucose into cells through glucose transporters. It also activates sympathetic nervous system increasing CO (Jellinger, 2007; Deedwania, 2011). Unfortunately, insulin resistance is a common medical diagnosis for people who have hypertension and diabetes. Lack of glucose uptake by cells can have a plethora of health consequences. For example, insulin resistance could amplify the synthesis of proinflammatory cytokines and impair the fibrinolysis signaling pathway (Cheung and Li, 2012). Free fatty acids from adipocytes are known to cause insulin resistance in the liver, endothelial cells, and skeletal muscles (Boden, 2008).

2.7 Hypertension and Renal Damage

Hypertension damages blood vessels of the systemic circulation and those that are proximal to the kidneys. Specifically, chronic hypertension can narrow the inner diameter of blood vessels through vascular remodeling. It could also weaken the smooth muscle cells rendering them less contractile. Finally, it could stiffen or harden

the blood vessels compromising their elastic property (Franklin, 2005). Inflammatory cytokines may be recruited to damaged lumen that eventually forms a plaque. This narrowing of the blood vessels could lead to higher pressure (increase in vascular resistance) and increase cardiovascular risk. Moreover, hypertension inflicts similar damage to the vessels near and inside the kidney depriving the renal system the essential nutrients and oxygen (Textor, 2017). Conversely, the damaged kidney will not able to excrete salt and water efficiently. As a result, the blood volume would increase which means the CO will increase as well (Salem, 2002). Summation of the renal and vascular damage will culminate in amplified hypertension.

Chronic kidney disease (CKD) is characterized by continuous kidney damage that results in a weakened kidney function marked by the presence of albumin in the urine. It is one of the major health issues in the United States (Coresh et al., 2007) and globally (Hill et al., 2016). One reason for the growing prevalence of CKD is the prevalence of hypertension. Most patients with CKD are hypertensive. The strong correlation between these two conditions leads to end-stage renal disease (ESRD) that can only be clinically addressed via dialysis or kidney transplant. In addition, CKD is accompanied by a number of risk factors such as anemia, and dyslipidemia that could make CKD fatal (Mennuni et al., 2014).

Race, gender, and age are confounding factors in the hypertension driven CKD. Hypertension in African American CKD patients is higher than African American women and Caucasians (both sexes) (Duru et al., 2008). On the other hand, old age increases the risk for death compared to ESRD (O'Hare et al., 2007), which is not that surprising.

Nevertheless, hypertension is linked to CKD in all ages, but shows stronger association in younger patients (Islam et al., 2009).

2.8 Oxidative Stress and Inflammation in Hypertension

There is a causal relationship between hypertension and oxidative stress as well as between hypertension and inflammation. In other words, hypertension causes oxidative stress and inflammation; however, the inverse is true as well, i.e., oxidative stress and inflammation can cause hypertension (Vaziri and Rodriguez-Iturbe, 2006; Vaziri, 2008). There is limited evidence that suggests hypertension as a cause for oxidative stress. Few rat studies indicate that oxidative stress in the blood vessels near the abdominal aorta coarctation is due to shear stress that result from high blood pressure (Barton et al., 2001; Sindhu et al., 2005; Vaziri and Ni, 2005). Nevertheless, human studies are not consistent regarding this although antihypertensive drugs are reported to reduce oxidative stress in addition to lowering blood pressure (Grossman, 2008). In contrast, there are plenty of animal and human (only some) studies validating the notion that oxidative stress is one of the main causes of hypertension. Some of the mechanisms are: increases proliferation of vascular smooth muscle cells leading up to hypertrophy and collagen accumulation that thickens the wall and narrows the vascular opening increasing vascular resistance; and disrupts the nitric oxide-mediated vasorelaxation system by damaging the endothelium. This relationship is further demonstrated by the reduction in oxidative stress and to some extent that of blood pressure following antioxidant treatment (Galley et al., 1997; Akpaffiong and Taylor, 1998; Rodriguez-Iturbe et al., 2003).

One of the consequences of hypertension is the induction of vascular damage accompanied by inflammation. Angiotensin II (Ang II) is among several factors that drive hypertension-related vascular damage. In this process, Ang II orchestrates the recruitment of inflammatory cytokines (e.g., TNF-α, IL-1,6,15)(Bautista et al., 2005; Kaibe et al., 2005; Leibowitz et al., 2005) and chemokines (e.g., MCP-1)(Suzuki et al., 2003) in addition to participating in the adhesion of immune cells to the walls of the endothelium . In addition, reactive oxygen species participate in exacerbating the Ang II-induced inflammation and vascular damage (Ruiz-Ortega et al., 2006).

The role of immune system, via inflammation, in the development of hypertension has been extensively explored (White and Grollman, 1964; Harrison, 2014; Drummond et al., 2019). Although there are some early studies of this relationship (Okuda and Grollman, 1967), more attention is given in the recent times to the contribution of immune cells to evoke or amplify inflammation of blood vessels in the central and peripheral circulation resulting in hypertension. Cytokines such as IL-17 and TNF-α have been implicated in causing hypertension. For example, IL-17a-deficient mice did not become hypertensive despite exposure to Ang II (Madhur et al., 2010). Moreover, T cell infiltration to the aorta was absent. Similarly, IL-17a is indicated in endothelial damage and hypertension (Nguyen et al., 2013). Others have also shown that using an antagonist for TNF-α (Etanercept) effectively reduces blood pressure (Tran et al., 2009; Venegas-Pont et al., 2010). Macrophages have also been known to play some role in the pathogenesis of hypertension (Wenzel et al., 2016; Huang et al., 2018).

2.9 Vascular Dysfunction Related to Hypertension

Endothelial cells in the inner lining of blood vessels are prone to hypertension-related damage (Widlansky et al., 2003). Endothelial dysfunction is marked by inflammation (Zhang, 2008), narrowing of the lumen (atherosclerosis) (Gimbrone and Garcia-Cardena, 2016), and increased vascular tone (Loscalzo, 1995). Association of inflammation and atherosclerosis with vascular damage were discussed in earlier part of this introduction, and this paragraph will focus on the impairment of vascular tone. Endothelium is a dynamic structure of the blood vessel which releases both vasoconstrictive and vasodilatory factors. The vasoconstrictive factors include thromboxane (TXA_2), and endothelin-1 (ET-1) through ETa receptor in smooth muscle cells whereas the vasodilatory factors includeET-1 through ETb in endothelial cells, nitric oxide (NO), endothelium derived hyperpolarizing factor (EDHF), and prostacyclin (PGI2) (Sandoo et al., 2010). These factors along with vasoconstrictive neurotransmitters (norepinephrine and ATP) released from the presynaptic terminal control the overall vascular tone. There is also myogenic tone which is a pressure induced tone in arterioles and many resistance arteries. This is very important in skeletal muscle which makes up a large fraction of the arterial circulation and peripheral vascular resistance. However, the net vascular tone increases when the endothelial cells are damaged reducing the level of NO. As a result, vascular resistance increases contributing to increase in blood pressure (Nadar et al., 2004).

2.10 Current Treatments for Hypertension

Controlling hypertension reduces the risk for cardiovascular disease, type 2 diabetes, and kidney damage. Besides lifestyle change, there are antihypertensive

drugs commonly used to treat for hypertension. These drugs are divided into five main categories: diuretics, angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, ang II receptor antagonists and calcium channel blockers (CCBs). Note that there are other classes of drugs as well: alpha-adrenergic receptor blockers, renin inhibitors, direct acting vasodilators, and centrally acting drugs (Laurent, 2017). Depending on individual patient's condition, one or a combination of these drugs are utilized (Nguyen et al., 2010).

Diuretics are drugs that remove salt and water through urine. There are three kinds of diuretics: thiazide, loop, and potassium-sparing. They differ in their mechanism and location of action in the nephron. Thiazide, loop, and potassium-sparing diuretics inhibit reabsorption of Na^+ in the early distal tubule, ascending limb of the loop of Henle, and late distal tubule and collecting duct, respectively (Laurent, 2017). ACE inhibitors prevent ACE from converting Ang I to Ang II systematically and in endothelial cells in blood vessels (Herman and Bashir, 2019). Beta blockers decrease CO by blocking β1-adrenergic receptor in the cardiac muscle (Frishman and Alwarshetty, 2002). Ang II receptor blockers exert their effect by blocking a subtype I of its receptor (AT_1) located different sites in the kidney, cardiac and vascular cells, and in some regions of the brain (Laurent, 2017). Calcium channel blockers antagonize L-type calcium channels resulting in smooth muscle relaxation in blood vessels as well as relaxation in cardiac myocytes (Kohlhardt and Fleckenstein, 1977) (Table 3).

Alpha1-adrenergic antagonists such as prazosin inhibit the action of NE to reduce vascular tone. Renin inhibitors such as aliskiren act by inhibiting renin activity, and hence disrupting the conversion of angiotensinogen to ang I (Rahuel et al., 2000).

Direct acting vasodilators such as minoxidil activate the ATP-dependent sarcoleminal potassium channels in the arterial smooth muscle cells resulting in vasorelaxation (Mannhold, 2004). Centrally acting drugs such as clonidine (alpha2- adrenergic receptor agonist) reduce sympathetic nerve outflow from the brainstem (Schmitt and Fenard, 1973).

Table 3. Commonly used antihypertensive drugs and their mechanisms of action (Laurent, 2017).

Drug Class	**Sub-classes**	**Example**	**Target**	**Mechanism of Action**
Diuretics	Thiazides	Hydrochlorothiazide	Early convoluted distal tubule	Inhibit Na^+ and Cl^- reabsorption
	Potassium sparing	Amiloride	Late distal tubule and collecting duct	Inhibit Na^+ reabsorption
ACE inhibitors	---	Enalapril	ACE	Prevent conversion of Ang I to Ang II
Beta blockers	Non-selective	Propranolol	Cardiac myocytes and VSMCs	Binds to β-AR and blocks activity
	β1-selective	Atenolol	Cardiac myocyte	Binds to β1-AR and blocks muscle contraction
	With α-blocking activity	Carvedilol	Cardiac and vascular cells	Binds to α- and β-AR and blocks muscle contraction
Ang II receptor blockers	---	Losartan	Endothelial cells in kidney and blood vessels	Binds AT_1 and leads to vasodilation
CCBs	Dihydropyridines (DHPs)	Nifedipine	VSMCs	Blocks L-type Ca^{2+} channels
	Non-DHPs	Verapamil	Cardiac myocytes	Blocks L-type Ca^{2+} channels

3.0 Obesity-associated Hypertension

3.1 Mechanisms of Obesity-related Hypertension

A 2009 report from the American Heart Association revealed that close to 75% of hypertension is linked to obesity (Landsberg et al., 2013).Obesity and hypertension pose health risks when they exist individually; however, when they co-exist, then the cardiovascular risk, morbidity and mortality rate are highly increased making therapeutic results more difficult to achieve. Therefore, it is important to understand the mechanisms by which obesity is associated with hypertension. Here some of the main mechanisms implicated in obesity-associated hypertension: overactivation of the sympathetic nervous system, renal dysfunction, altered hormonal function, vascular and endothelial changes, and increased engagement of the immune system (Fig. 4).

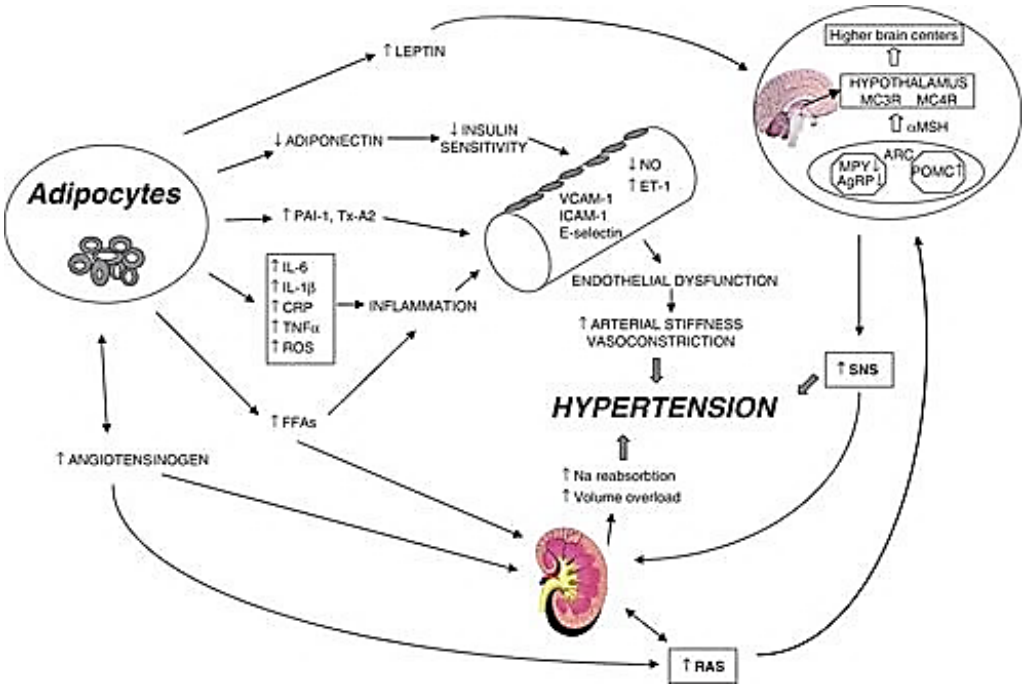


Figure 4. Mechanisms of obesity-associated hypertension. IL-6, interleukin-6; IL-1β, interleukin-1β; CRP, C-reactive protein; TNFα, tumor necrosis factor-α; ROS, reactive oxygen species; FFAs, free-fatty acids; NO, nitric oxide; ET-1, endothelin-1; RAS, renin-angiotensin system; Ang II, angiotensin II; SNS, sympathetic nervous system (Kotsis et al., 2010).

The activity of the sympathetic nervous system (SNS) can be assessed using either a direct approach with a muscle microneurography or indirectly by measuring plasma NE level. It has been shown that muscle sympathetic nerve activity increased in obese individuals (Grassi et al., 1995). Moreover, HFD elevated plasma NE level in addition to activating the adrenergic system in the vascular system leading to increased SNA and blood pressure (Landsberg and Krieger, 1989). HFD-fed experimental animals had increased level of hypothalamic tyrosine hydroxylase and upregulation of adrenergic receptors (Coatmellec-Taglioni and Ribiere, 2003; Rocchini et al., 2004). Adrenergic blockage produced greater reduction in hypertension in obese versus non-obese individuals (Wofford et al., 2001). Some of the factors driving the obesity-related sympathetic overdrive are elevated circulating free fatty acids (FFAs), insulin, leptin, Ang II, and dysfunctional baroreceptor reflex (Kotsis et al., 2010) (Fig 4.).

Adipocytes are the source of FFAs, reactive oxygen species (ROS) and several inflammatory substances, such as interleukins, TNF-α, and C-reactive protein (Fig. 4). Circulating FFAs interact with α-adrenergic receptors which increases vascular tone although this mechanism is not well understood (Stepniakowski et al., 1995). They also interact with the Na^+/K^+ pump that in turn alter downstream signaling pathways resulting in increased production of reactive oxygen species (ROS) (Oishi et al., 1990; Inoguchi et al., 2000). In addition, FFAs are suggested to activate protein kinase c, which is involved in smooth muscle cell contraction (Blobe et al., 1993). Finally, FFAs may be directly involved in altering the function of ions channels in vascular smooth muscle cells (Ordway et al., 1991).

The mechanism by which insulin elevates sympathetic activity is not well established. Nevertheless, one study suggested that insulin may impact the sympathetic nervous system through the anteroventral third ventricle hypothalamic region of the brain (Muntzel et al., 1995). Another study from obese mice demonstrated that insulin may act via phosphoinositol-3 kinase signaling pathway in activating the sympathetic nervous system (Morgan and Rahmouni, 2010). Insulin may also increase sympathetic activity by suppressing the inhibitory action of neuropeptide Y in the paraventricular nucleus (Cassaglia et al., 2016). In contrast, insulin infusion in humans showed increase in sympathetic activity that did not translate to increase in blood pressure (Anderson et al., 1992).

As previously mentioned, leptin is a peptide hormone released from adipocytes, and it is essential in regulating energy balance in the body. Leptin has receptors in the hypothalamus and brain stem that when bound increases the sympathetic activity and energy expenditure while suppressing the desire to eat (Rahmouni et al., 2005; Hall et al., 2010). More evidence for the link between leptin and the SNS is shown when antagonism of adrenergic receptors eliminated the effect of leptin (da Silva et al., 2009). Further, leptin has been shown to increase the plasma NE level which indirectly confirmed its influence on the sympathetic activity (Satoh et al., 1999).

Ang II is closely associated with increase in sympathetic nerve activity in the central and peripheral nervous systems. In the central nervous system (CNS), the effect of Ang II on blood pressure is manifested through the stimulation of the SNS, release of vasopressin and suppression of baroreflex system (Phillips, 1987; Phillips and Sumners, 1998). Ang II receptor (AT_1) is localized in the paraventricular nucleus of the

hypothalamus. Activation of this receptor is the onset of sympathetic nerve signal conducted to nerve terminals on the vasculature. In addition, the activated AT_1 induces the release of vasopressin (also known as antidiuretic hormone, ADH) which is another vasoconstrictor (Reaux et al., 2001). Vasopressin increases vascular resistance in the blood vessels (Cowley, 1988) as well as acts on the collecting duct in the nephron of the kidney resulting in water retention (Cuzzo and Lappin, 2019). Ang II also inhibits the baroreceptor input from medulla oblongata. By doing so, Ang II increases the systemic blood pressure.

In the peripheral system, Ang II modulates vascular tone, and stimulates aldosterone release (this will be covered in depth in the next section). Ang II, via AT1, receptor in the vascular smooth muscle cells evokes vasoconstriction (Vukelic and Griendling, 2014). Further, Ang II leads to vascular smooth muscle cell hypertrophy because of increase in the production of extracellular matrix proteins, like collagen and fibronectin (Geisterfer et al., 1988; Griffin et al., 1991). This, in turn, can lead to vascular stiffening rendering the blood vessels highly resistant (Bhatta et al., 2015). Ang II has also been implicated in the development of atherosclerosis (Weiss et al., 2001). Moreover, Ang II is implicated in the proliferation of vascular smooth muscle cells (Escobar et al., 2004). The sum of these insults by Ang II on the vasculature leads to hypertension.

Normally, baroreceptors from the aortic arch and carotid sinus mediate sympathetic suppression and parasympathetic activation in response to high blood pressure. There are several causes for baroreceptor dysfunction, including trauma, surgery, brainstem stroke, tumor development, irradiation, afferent sensory neuropathy,

and genetics (e.g., hypertension-bradydactyly syndrome) (Ketch et al., 2002). Note that baroreflex impairment can occur in obese subjects without increased blood pressure (Kotsis et al., 2010). In addition, clinical diagnosis for baroreflex dysfunction is presented in temporary hypertension crisis due to previous surgical procedure (Ketch et al., 2002) or in permanent state of high blood pressure resulting from disruption in the input or output circuit.

3.2 Sex Difference in Obesity-associated Hypertension

The recognition for the need to understand sex-specific mechanisms in obesity-associated hypertension has been long overdue since such knowledge will pave a way for the development of better therapeutics for hypertension. There is a consensus that women have higher rate of obesity than men throughout the world (Yun et al., 2006; Ogden et al., 2015; Flegal et al., 2016; Faulkner and Belin de Chantemele, 2018). Both obesity and hypertension increase the risk for cardiovascular disease, and metabolic disorders such as type 2 diabetes. Lean women compared to lean men are protected from hypertension during premenopausal years, but such protection is not present in postmenopausal women (Burt et al., 1995; Faulkner and Belin de Chantemele, 2018). The cardioprotective role that estrogen is known to play is limited to lean premenopausal women. One reason for this may be that the higher level of leptin in obese women overrides the effect of estrogen (Castracane et al., 1998). Some literature suggests that there may even be an interaction between leptin and estrogen rendering the later ineffective (Gao and Horvath, 2008; Fungfuang et al., 2013). Testosterone has also been implicated to play some role in this process in obese women although the mechanism is not clear (Navarro et al., 2015). Contrary to the lean subjects,

hypertension is strongly associated with obesity in women than men (Wilsgaard et al., 2000; de Simone et al., 2006; Fujita and Hata, 2014; Sampson et al., 2014). This relationship is also reflected in the higher health risk with obesity-related hypertension in women versus men (Mosca et al., 2011), and that obesity-related hypertension is more challenging to clinically control in women than in men (Kim et al., 2006; Gudmundsdottir et al., 2012).

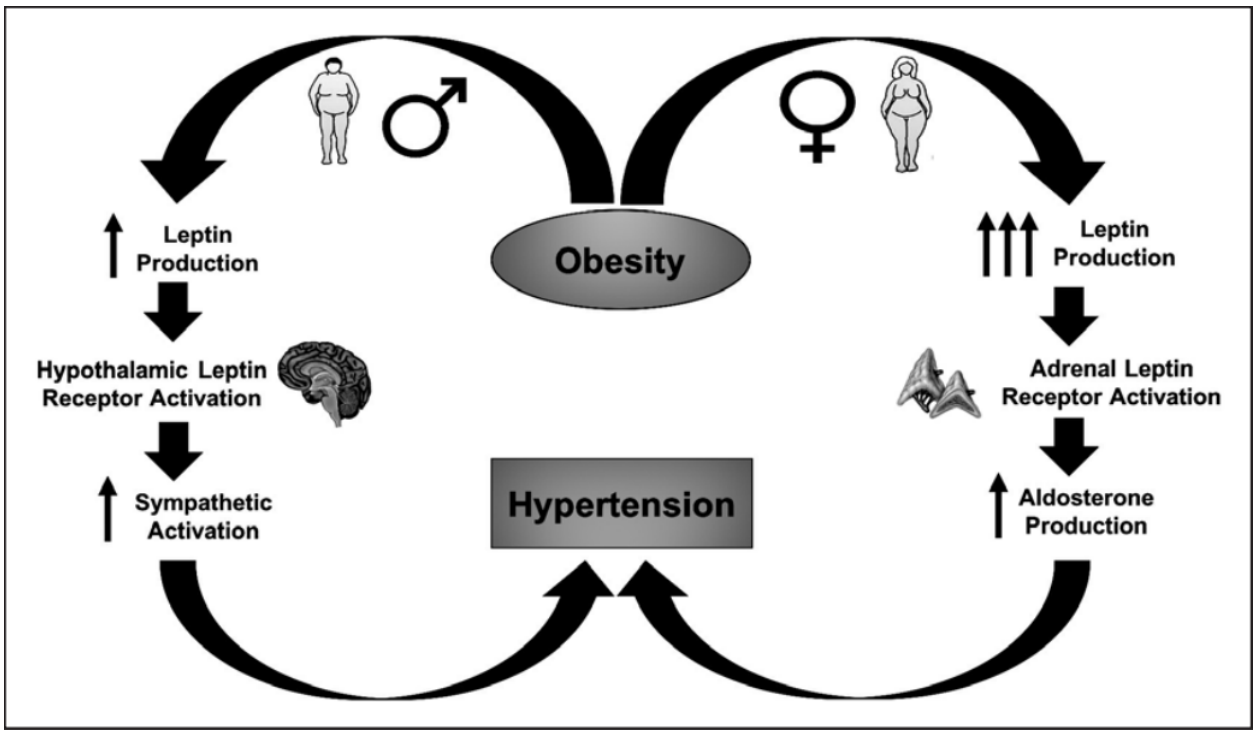


Figure 5. Sex-specific mechanisms in leptin-induced hypertension. In men, increase in leptin production from the adipose depot engages hypothalamic receptors at the CNS leading to increase in sympathetic activity and hypertension. In obese women, increase in leptin evokes adrenal activation followed by increase in aldosterone level that leads to increase in renal salt and water reabsorption, blood volume, and hypertension (Faulkner and Belin de Chantemele, 2018).

Leptin may be required in order to develop hypertension in obese male and female experimental animals (Belin de Chantemele et al., 2011; Wang et al., 2014). Subcutaneous adipose tissue releases more leptin than visceral adipose tissue

(Montague et al., 1997; Van Harmelen et al., 1998). Women in general have more subcutaneous adipose tissue than visceral tissue in comparison to men (Taylor et al., 2010). Therefore, obese women have higher level of leptin than obese men (Van Harmelen et al., 1998; Hellstrom et al., 2000). Not only is there sex difference in the leptin level but also in the mechanism by which leptin induces hypertension. In men, leptin binds and activates its receptors (leptin receptors) in the hypothalamus which in turn activates the sympathetic nerves resulting in increased blood pressure (Belin de Chantemele et al., 2009; Hall et al., 2010). In contrast, leptin binds to adrenal leptin receptors in women (Huby et al., 2015; Faulkner et al., 2018). Consequently, the activated adrenal leptin receptors increase the production of aldosterone. Aldosterone, through interaction with the mineralocorticoid receptor, increases water and salt retention in the nephron elevating blood volume and blood pressure (Goodfriend, 2006) (Fig. 5).

The renin-angiotensin-aldosterone system (RAAS) is important in regulating the cardiovascular system and fluid homeostasis via the kidneys. This system is modulated by sex hormones, namely estrogen, and testosterone. Estrogen has been associated with decrease RAAS activity whereas testosterone increases the RAAS activity (Komukai et al., 2010). There are not many studies showing sex differences in obesity-associated hypertension in the RAAS system. Although nascent, one evolving notion (Gupte et al., 2012) is that the vasoprotective effect of angiotensin (1-7) may be in play specifically in obese female. ACE2 converts AngI to Ang 1-7 whereas ACE1 converts AngI to AngII. Moreover, ACE2 level was increased in obese female while Ang II was decreased suggesting that obese females (at least in mice) are to some degree

protected from obesity-related hypertension by alternative pathways of RAAS. In addition, it has been shown that female spontaneously hypertensive rats have lower sensitivity to Ang II compared to males (Sullivan et al., 2010).

3.3 Influence of Age on Obesity-associated Hypertension

The association of body weight increase with increase in blood pressure is stronger and more prevalent in adults; however, this relationship can begin as early as before the teenage years (Falaschetti et al., 2010). With the current increasing trend in childhood obesity (Brady, 2017), age has become relatively less important as a factor for obesity-related hypertension. In this case, environmental factors such as lifestyle determine the risk factor for any individual regardless of age. Nevertheless, age remains a factor in increasing cardiovascular risk.

3.4 Racial and Ethnic Disparity in Obesity-associated Hypertension

The prevalence of obesity in adolescents is as follows, from highest to lowest (in %): Hispanics (23), African Americans (21), Caucasians (13), and Asians (10) (Claire Wang et al., 2011; Koebnick et al., 2012; Cheung et al., 2017). Among these groups, obesity-associated hypertension (in %) was the highest in the Hispanic (7.7) followed by the Caucasians (7.4), and it was similar level (4.5 and 4.6) in African Americans and Asians. Interestingly, the Caucasians with lower prevalence in obesity than African Americans have higher prevalence in obesity-related hypertension (Cheung et al., 2017). These differences may be influenced by lifestyle, social and economic status. It may also be affected by the diverse genetic makeup among the racial and ethnic groups (Stryjecki et al., 2018). Unfortunately, there is a lack of clear and consistent data from individual gene or genome wide association studies showing the relationship between

genes and obesity as well as obesity-related hypertension among the aforementioned groups.

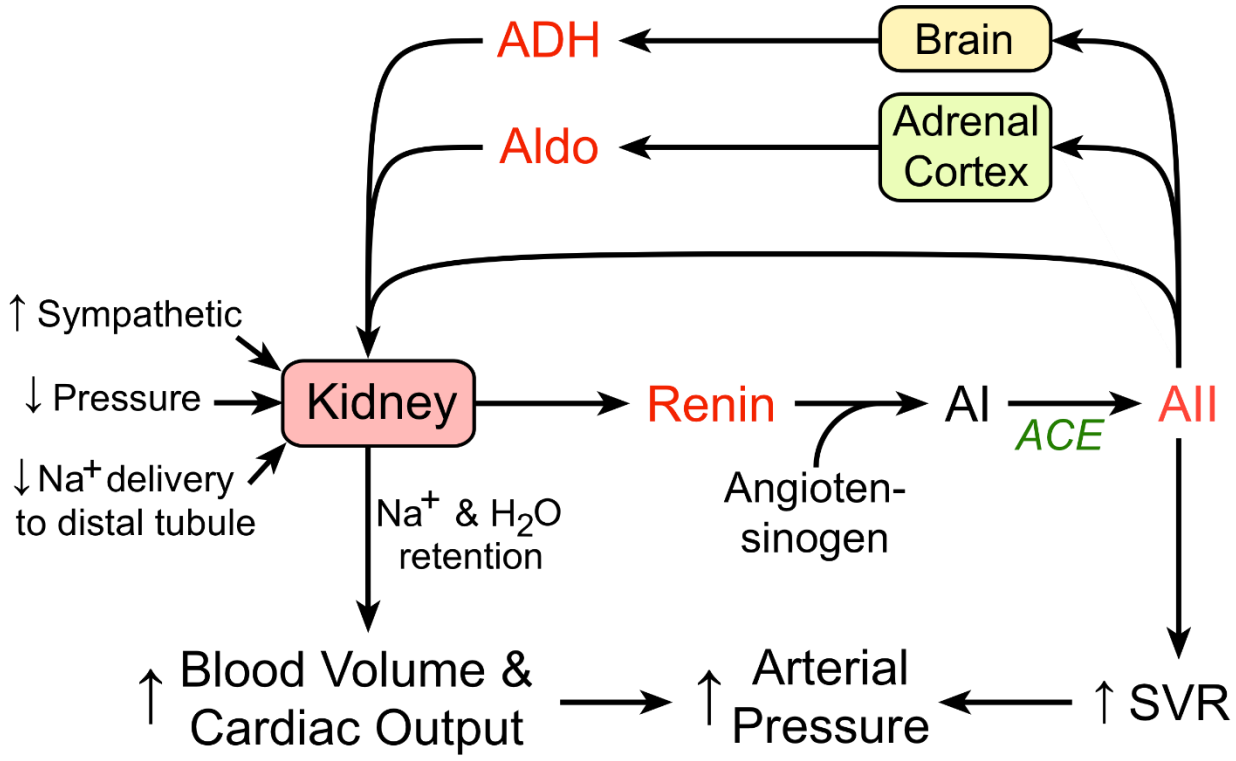


Figure 6. The role of kidney in the pathogenesis of hypertension. Increase in sympathetic activation, and renal sodium and water retention driving increase in CO and stroke volume culminating in blood pressure elevation (Klabunde, 2012b).

4.0 Role of Sympathetic Nervous System in Hypertension

4.1 Central and Peripheral Control of Blood Pressure

The sympathetic nervous system is a branch of the autonomic nervous system which plays an essential role in the homeostasis of blood pressure. It controls blood pressure mainly through the heart, kidney, and splanchnic circulation(Triposkiadis et al., 2009; Kannan et al., 2014; Sheng and Zhu, 2018). Sympathetic nerves projecting from medulla oblongata, down the spinal cord to the heart, which when activated increases heart rate. This activity is modulated by the baroreflex system involving the parasympathetic nerve fibers. The effect of the baroreflex is for a short term; however, the sympathetic innervation has alternative pathways to control blood pressure.

4.2 Renal Sympathetic Nerves and Hypertension

Renal sympathetic nerves refer to the sympathetic nerves that link the CNS to different parts of the kidneys. Renal sympathetic nerves modulate renal activities, such as glomerular filtration rate, sodium and water balance, and blood flow (Coffman, 2014). When renal sympathetic nerve activity (RSNA) increases, the kidneys malfunction leading to hypertension (Sata et al., 2018) (Esler and Guo, 2017). Renal contribution to hypertension involves four mechanisms: 1) a decrease in glomerular filtration, 2) an increase in sodium and water reabsorption, 3) a decrease in blood flow, and 4) an increase in renin. These alterations are exacerbated by obesity (Hall et al., 1999).

Norepinephrine and ATP released from the sympathetic nerves constrict blood vessels restricting blood flow or renal perfusion as well as reducing glomerular filtration rate. In addition, adrenergic receptors located in the proximal tubule mediate sodium and water reabsorption. Consequently, blood volume increases which means an

increase in CO and blood pressure (Hadtstein and Schaefer, 2008). The release of renin from the juxtaglomerular apparatus activates the RAAS (Hsueh and Wyne, 2011). The two products of an activated RAAS are Ang II and aldosterone that are involved in vasoconstriction, and water reabsorption, respectively. The combined assault of the renin, Ang II, and aldosterone culminates in kidney damage, hypertension, and cardiovascular disease. To minimize the impact of overactive RSNA, renal denervation techniques using a catheter or radiofrequency have provided promising outcomes in lowering blood pressure, and the later technique is at its early clinical trial stage (Singh and Denton, 2018). In addition, some studies demonstrated the effectiveness of the catheter-based technique in lowering blood pressure in resistant hypertension (Esler et al., 2014).

4.3 Sympathetic Regulation of Mesenteric Circulation

Splanchnic circulation refers to the circulatory system involving the small intestines, liver, colon, pancreas, spleen and stomach (Harper and Chandler, 2016). This circulation receives close to 30 % of CO, thereby making it an important participant in maintaining normal blood pressure (Morato et al., 2008; Harper and Chandler, 2016). Mesenteric arteries (MA) and veins (MV) supply and take away blood to and from the small intestines, respectively. They have an outermost layer known as adventitia, followed by smooth muscle cells, and the endothelium (innermost structure). The main difference between these vessels is that MA has more layers of smooth muscle cells than veins (dela Paz and D'Amore, 2009). Functionally, MA is referred as resistance artery because there is a significant drop in blood pressure as you pass through these arteries into the gut. On the other hand, MV is referred as capacitance vessel since it

holds larger volume of blood. Mesenteric veins are known to have greater compliance than MA, i.e., they have the ability to stretch to accommodate more blood with minimal increase in blood pressure(Nilsson, 1985).

The mesenteric vasculature and its microcirculation are a dynamic system that is controlled by intrinsic, extrinsic, and humoral mechanisms. The intrinsic mechanism maintains appropriate oxygen supply to the blood vessels. Lack of sufficient oxygen results in vasodilation, and vice versa (Granger and Kvietys, 1981). Moreover, the little myogenic tone that they have allows them to keep blood flow to a basal level in spite of mechanical changes such as stretch that vascular cells experience. This is mediated by voltage-gated calcium channels (Takala, 1997).

Both, MA and MV are innervated by postganglionic sympathetic nerves that are project from celiac ganglia. These nerves terminate at the adventitia proximal to the vascular smooth muscle cells (Hsieh et al., 2000). Together, they form a neurovascular junction whereby the presynaptic nerves release NE and ATP which are important in regulating vascular tone by binding to the adrenergic and purinergic receptors in the VSMCs, respectively (Stephens and Heagerty, 1994; Burnstock, 2009). Therefore, sympathetic control of the mesenteric blood vessels is essential in the distribution of blood volume in the small intestine. Nitric oxide is a powerful vasodilator synthesized in the endothelium but modulates the vasoconstrictive effect of the sympathetic transmission on the smooth muscle cells (Chadha et al., 2011). This mechanism is often altered in hypertension (Sullivan et al., 2002).

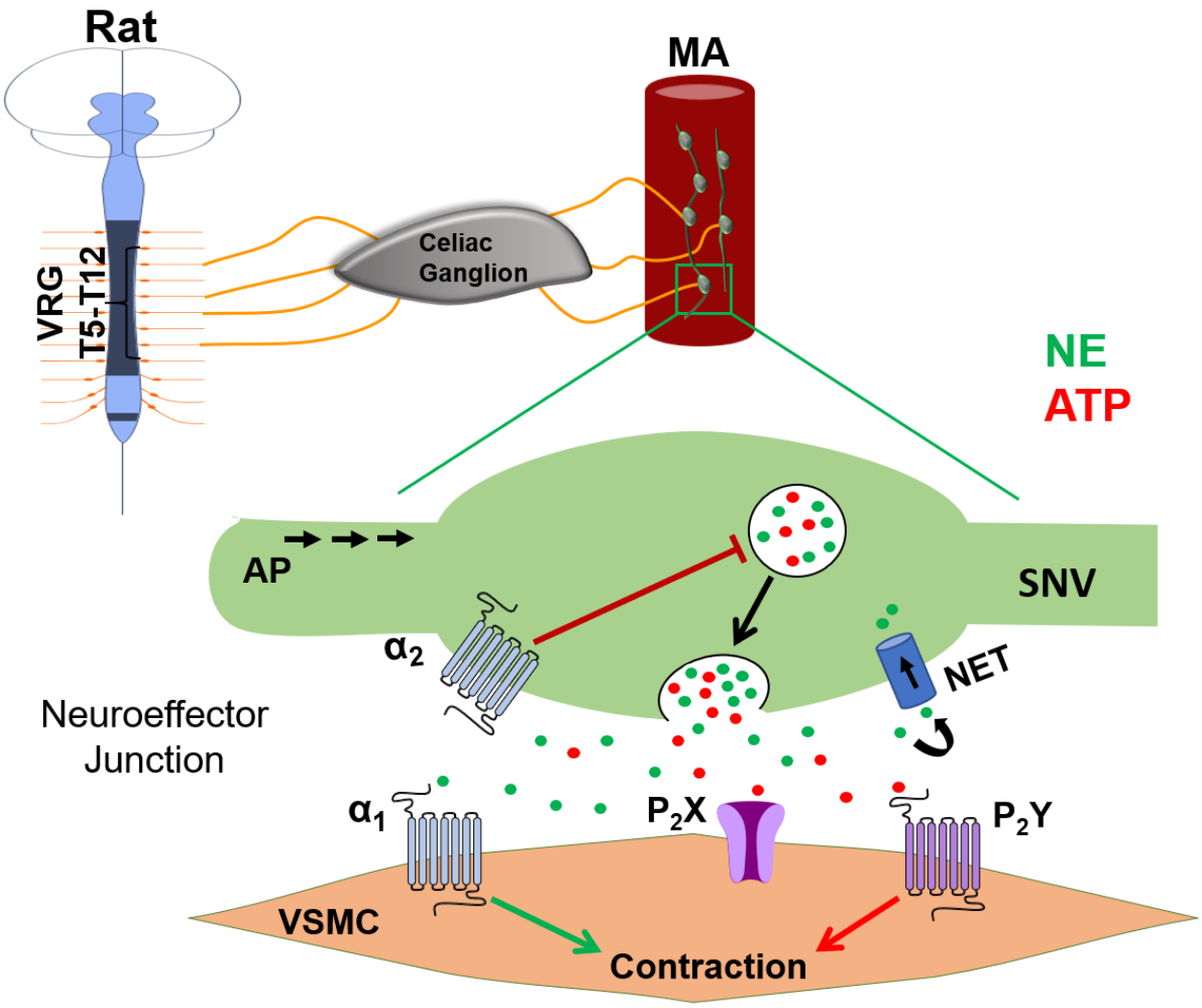


Figure 7. Sympathetic neurotransmission in MA. Sympathetic nerves project out from celiac ganglion and innervate MA. Axonal action potential (AP) causes NE (green) and ATP (red) release from sympathetic nerve varicosity (SNV) into the neuroeffector junction. The number of neurotransmitters in the neuroeffector junction is regulated through negative feedback inhibition by α2 adrenergic receptor, and via reuptake mechanisms by norepinephrine transporter (NET). NE binds to α_1 and ATP binds to P_2X and P_2Y receptors in the vascular smooth muscle cell (VSMC) resulting in muscle contraction.

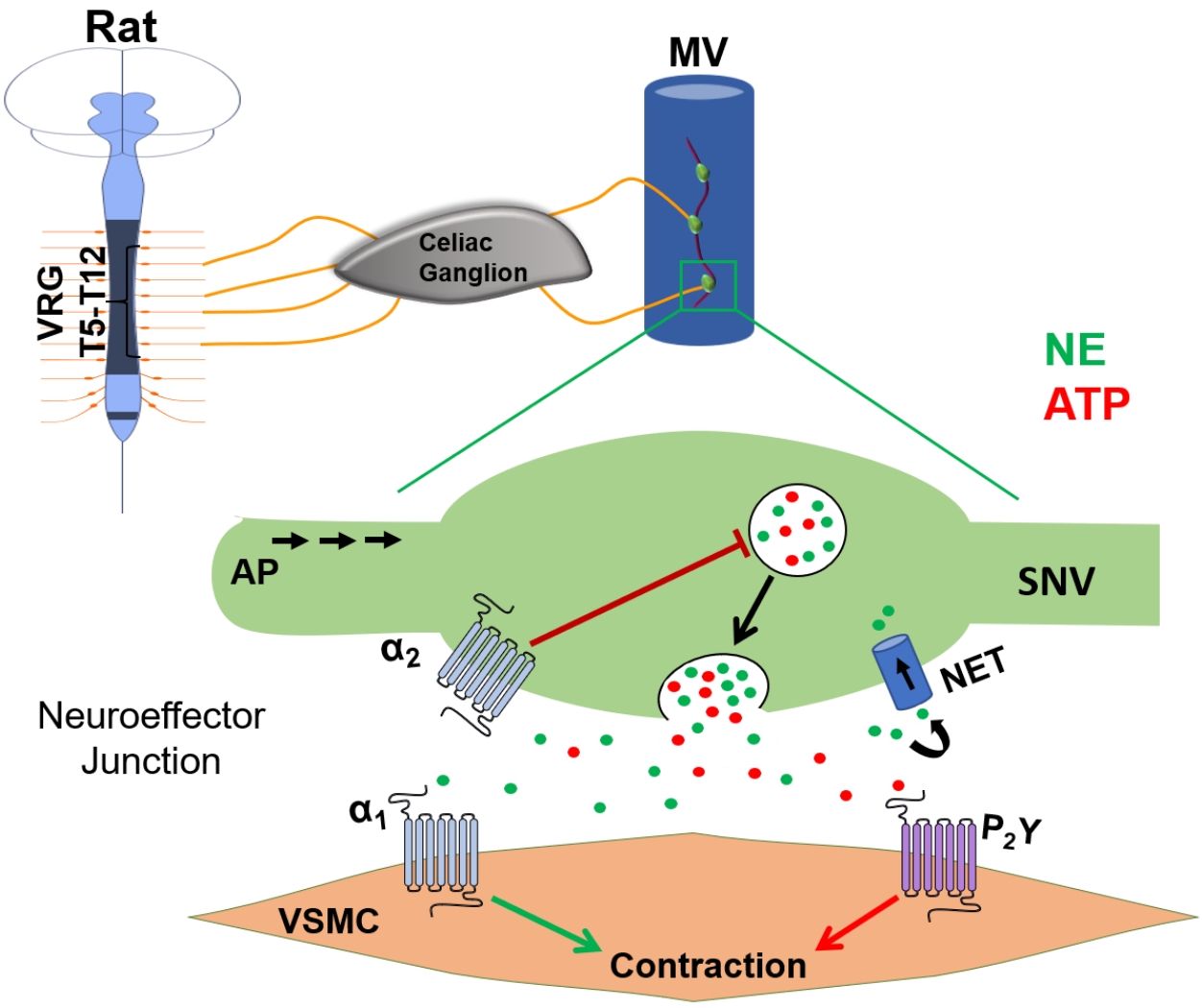


Figure 8. Sympathetic neurotransmission in MV. Sympathetic nerves project out from celiac ganglion and innervate MA. Axonal action potential (AP) causes NE (green) and ATP (red) release from sympathetic nerve varicosity (SNV) into the neuroeffector junction. The number of neurotransmitters in the neuroeffector junction is regulated through negative feedback inhibition by α2 adrenergic receptor, and via reuptake mechanisms by norepinephrine transporter (NET). NE binds to α_1 and ATP binds to P_2Y receptor in the vascular smooth muscle cell (VSMC) resulting in muscle contraction.

4.4 Sympathetic Neurotransmission in the Neuroeffector Junction

Vascular activity in the mesenteric blood vessels is under the control of the sympathetic nerve axonal terminals. The sympathetic nerves act on the vascular smooth muscle cells through two major mechanisms: adrenergic and purinergic neurotransmission. The adrenergic neurotransmission is mediated by NE whereas purinergic neurotransmission is mediated by ATP (Taylor and Parsons, 1989).

Adrenergic neurotransmission. The synthesis of NE in the axonal terminal begins with the conversion of tyrosine to DOPA by tyrosine hydroxylase (TH), which is a rate-limiting enzyme. Then, DOPA is converted to dopamine (DA) by another enzyme called DOPA decarboxylase. Subsequently, DA is transported into vesicles aided by vesicular monoamine transporter 2 (VMAT2; neuronal isoform) and converted to NE by dopamine β-hydroxylase (Kvetnansky et al., 2009). When an incoming action potential down the axon reaches the nerve terminal, it depolarizes the membrane causing calcium influx and release of NE from the vesicles by exocytosis (Esler et al., 1988; von Kugelgen et al., 1994). Norepinephrine binds to adrenergic receptors located in the membranes of vascular smooth muscle cells. They are alpha1-adrenergic receptor (α1-AR), α2-AR (Insel, 1989) and beta2-adrenergic receptor (β2-AR), and all of them are G-protein coupled receptors (metabotropic) that have seven membrane spanning domains. Nevertheless, activation of α1- and α2-ARs induce vasoconstriction whereas activation of β2-AR causes vasorelaxation (Tanaka et al., 2005). These receptors are found in both MA and MV.

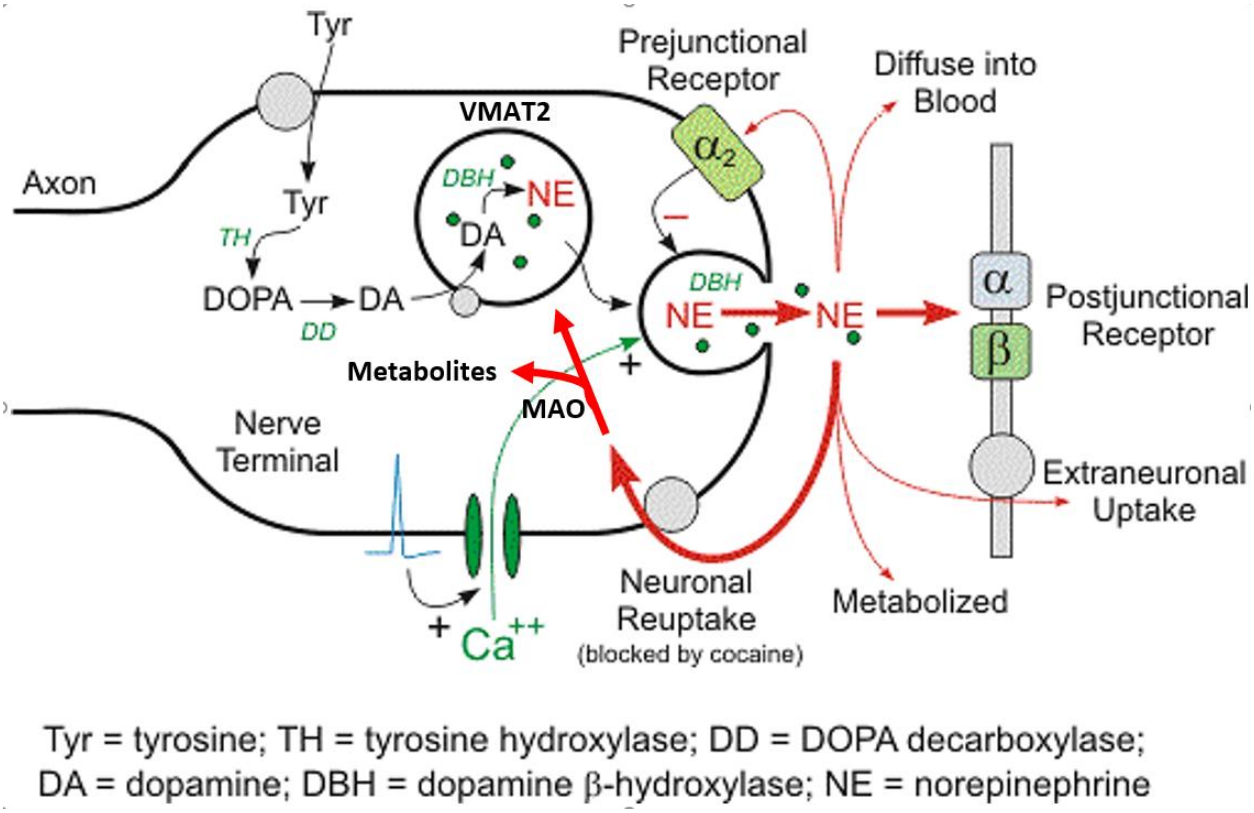


Figure 9. Synthesis, release, and clearance mechanisms for NE. Synthesis of NE involves Tyr, TH, DD, DA, and DBH. Once NE is released, it binds to its postjunctional receptors (α and β) activating downstream signaling pathways. Then, NE is cleared from neuroeffector junction through reuptake into VMAT2, diffusion and metabolism by MAO (Klabunde, 2012a).

When inactive, the α1-AR (on the cytosolic side) forms a complex with Gq-GDP. Binding of NE to the α1-AR changes the receptor's conformation and GTP is exchanged for GDP while releasing the G-protein (Gq). The Gq then activates phospholipase C (PLC), which in turn increases secondary messengers (inositol triphosphate (IP3)). Released IP3 binds to the sarcoplasmic reticulum causing an efflux of calcium ions. Voltage gated L-type calcium channels are also activated to allow calcium influx. Calcium binds to calmodulin and activates myosin light chain kinase (MLCK), which in turn phosphorylates MLC in the company of ATP. Phosphorylated MLC forms a cross bridge with actin filaments leading to vasoconstriction (Brozovich et al., 2016). Although to a lesser degree, α2-AR (linked to Gi proteins) also contribute to the vasoconstriction by decreasing intracellular cyclic AMP (cAMP) (Aburto et al., 1993). There are α2-AR (autoreceptors) in the presynaptic terminal as well and their main function is to regulate neurotransmitter release through a negative feedback mechanism (Giovannitti et al., 2015). The β2-ARs in the vascular smooth muscle cells are attached to a Gs-protein that increases intracellular cAMP. As a result, cAMP inhibits MLCK resulting in vasorelaxation (Brozovich et al., 2016).

Norepinephrine is cleared from the junctional space through different mechanisms. Close to 90% of NE is transported back to the nerve terminal via a norepinephrine transporter (NET) and transported into vesicular monoamine transporter 2 (VMAT2) (Iversen, 1971). Blocking of this transporter by cocaine results in sustained high-level junctional NE that can lead to hypertension. Some of the NE diffuse away into the circulation, and some NE metabolized by monoamine oxidase (MAO).

Approximately 5% of the NE is taken up by the postjunctional smooth muscle cells by a process known as extraneuronal uptake and broken down (Becker, 2012).

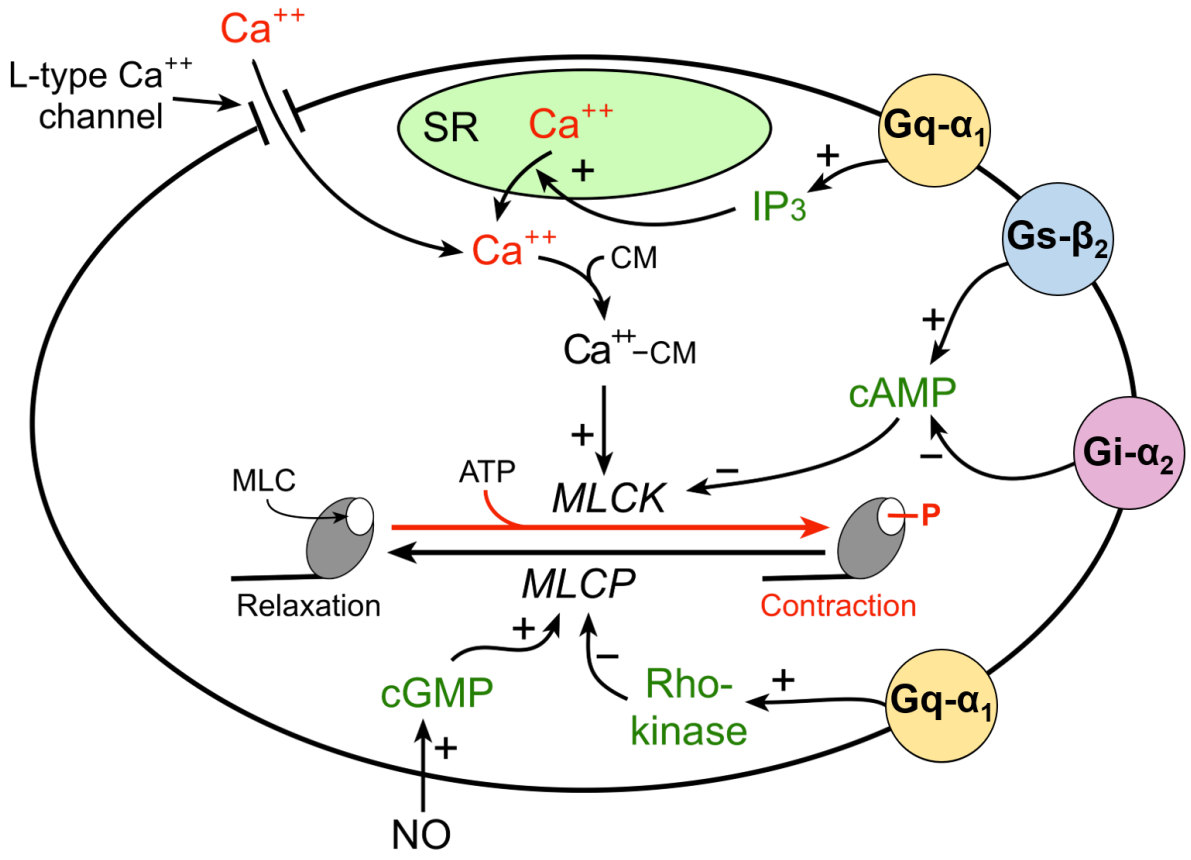


Figure 10. Schematic diagram showing adrenergic signaling pathways in the VSMC. Alpha 1 (α1) through IP3 induces calcium release from the sarcoplasmic reticulum (SR). This calcium along with the calcium influx from via the L-type calcium channel bind to calmodulin to activate MLCK and induce contraction. Further, α1 activates Rho kinase, which inhibits MLCP. Alpha 2 causes contraction by inhibiting cAMP. Beta 2 increases cAMP that inhibits MLCK to relax muscle. Moreover, Nitric oxide (NO) diffused from the endothelium activates cyclic GMP (cGMP) that activates MLCP to relax muscle. Figure modified from (Klabunde, 2012c).

Purinergic neurotransmission. Adenosine triphosphate (ATP) functions as a source of energy for cells throughout the body. It is also a neurotransmitter in the autonomic nervous system. Thus, ATP is important in maintaining hemodynamics of the mesenteric vasculature. Mitochondria residing in the nerve terminal synthesize ATP via the process of oxidative phosphorylation. Other systems such as the citric acid cycle and glycolysis also produce ATP, albeit in small amount. Then, the ATPs are transported into vesicles by a vesicular nucleotide transporter (VNUT). Note that the cytoplasmic ATP are not utilized for neurotransmission since they are in a 'bound form', i.e., linked with another compound and not free (Sperlagh and Vizi, 1996). Like NE, ATP is also released from its storage in response to membrane depolarization (Pankratov et al., 2007; Abbracchio et al., 2009). There is mounting evidence showing that NE and ATP can also be stored in the same vesicles as well as co-released by the same frequency of stimulation. This will be discussed in later chapters in more detail.

ATP binds to two types of receptors located on the postjunctional vascular smooth muscle cells. The first type is the P2X receptor which is a ligand-gated ion channel (ionotropic). The second type is P2Y receptor and this is a GPCR (metabotropic), which is slower than the P2X receptor. Smooth muscle cells from MA contains both P2X and P2Y receptors whereas MV only has P2Y receptors (Galligan et al., 2001). When ATP binds to the P2X receptor, the channel opens to let calcium and sodium ions move into the cell while potassium ion moves out. This depolarizes the membrane which activates the voltage gated L-type calcium channel and followed by calcium influx. Then, calcium binds to calmodulin triggering a sequence of events that lead to vasoconstriction. On the other hand, ATP-bound P2Y receptors change their

conformation (similar to NE-bound α1-AR) (Hattori and Gouaux, 2012). This, in turn activates the Gq-protein that activates the PLC. Activation of IP3 by PLC releases more calcium from the sarcoplasmic reticulum. The increased calcium binds to calmodulin and results in vasoconstriction (Bjorkgren and Lishko, 2016).

Pannexin is a type of ATP channel which is found in different tissues of the human body (Good et al., 2015), and in rats (Billaud et al., 2012) and mice (DeLalio et al., 2018). Pannexin1 is one of the three isoforms that is found in mesenteric smooth muscle cells although the involvement of pannexin1 in MV is not well understood (Li et al., 2015). It is a hexameric hemichannel that when activated by α1-AR, it opens up to release ATP from the vascular smooth muscle cytoplasm into the neuroeffector junction (Billaud et al., 2015; Chiu et al., 2017). Consequently, this further increase vasoconstriction by activating adjacent purinergic receptors.

Some studies suggest that ATP is co-released with nucleotidases, which breaks down ATP to ADP, AMP, and eventually to adenosine (Westfall et al., 2002; Gourine et al., 2009). Then adenosine binds to A1 adenosine receptors (A1Rs) localized in the presynaptic nerve terminal. The binding of adenosine to A1Rs is believed to activate the inhibition process for a NE release (Sangsiri et al., 2013).

4.5 Non-adrenergic and non-purinergic Sympathetic Neurotransmitters

Neuropeptide Y (NPY) is found in the central and peripheral nerves. It is synthesized and co-released (with NE and ATP) from the sympathetic nerves in the neuroeffector junction (Racchi et al., 1999). Like NE and ATP, NPY is a vasoconstrictor (Westfall et al., 1990). NPY receptors, Y1, are localized in the mesenteric vascular smooth muscle cells. These receptors are GPCR, specifically Gαq/11 that activate PLC

and then IP3 (similar to α1-AR) to induce vasoconstriction (Saraf et al., 2016; Tan et al., 2018). NPY-mediated activity has also been implicated in facilitating the NE or ATP induced vascular response (Racchi et al., 1997; Donoso et al., 2004). Moreover, NPY is associated with obesity and cardiovascular disease similar to NE and ATP (Beck, 2006; Zhu et al., 2016).

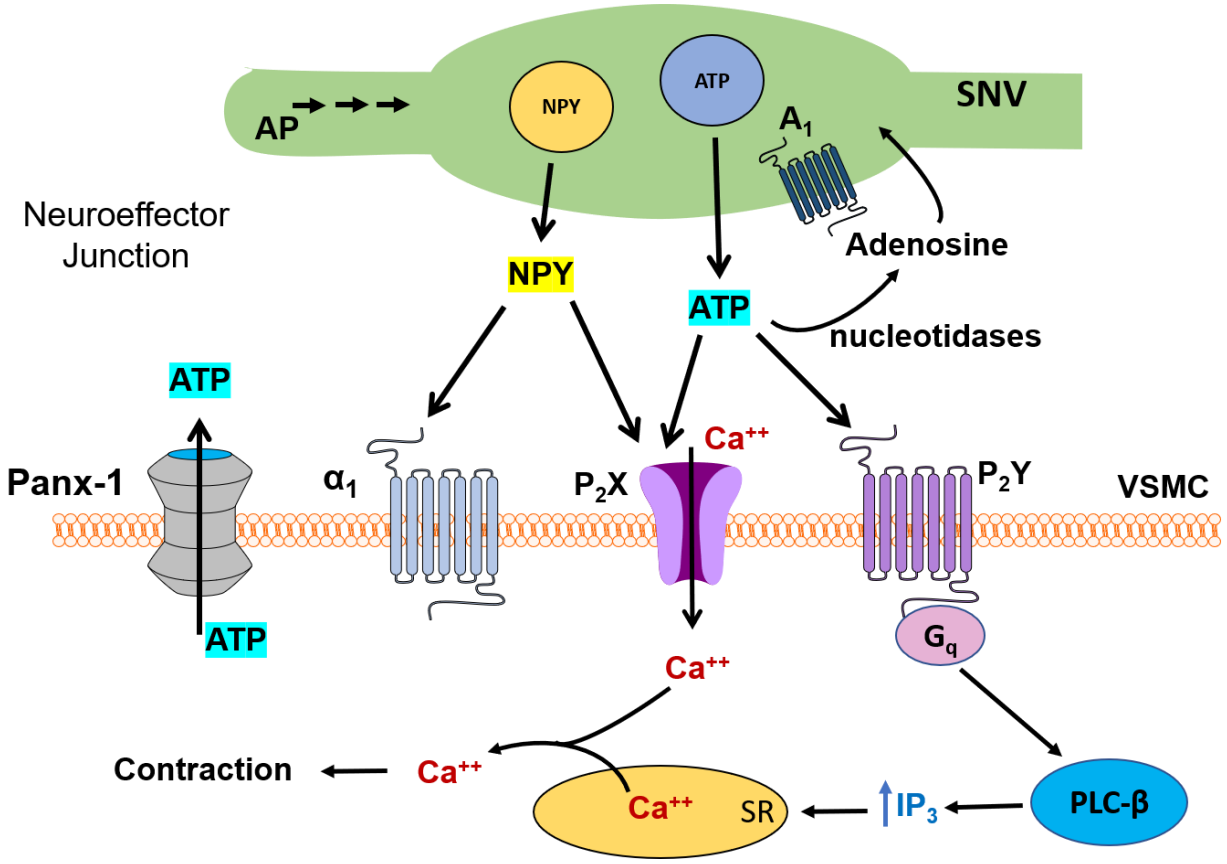


Figure 11. Depiction of purinergic signaling pathways in VSMC. ATP binds to P_2X (ionotropic) and P_2Y (metabotropic) receptors to induce vasoconstriction. Activated P_2Y receptor activates PLC-β that in turn increases IP_3. Increased IP3 releases calcium from SR storage leading to muscle contraction.

4.6 Sensory Modulation of Vascular Response

Sensory or afferent neurons in the mesenteric vasculature release three neurotransmitters, namely: calcitonin gene-related peptide (CGRP), substance P (SP), and ATP. The first two are powerful vasodilators of the smooth muscle cells, hence playing an important role in maintain vascular tone (Rubino and Burnstock, 1996), and keeps blood pressure within the physiological level (Wimalawansa, 1996). CGRP is synthesized in the dorsal root ganglia (DRG) and stored in the sensory nerves proximal to the mesenteric smooth muscle cells. Its receptors are located in the media and intima regions of the blood vessels. CGRP receptors (CGRP1)(Brain and Grant, 2004) are GPCRs that when activated by CGRP increase cAMP (Watson et al., 2002). In addition, some studies suggest that CGRP may activate ATP-dependent potassium channels in the smooth muscle cells (Wimalawansa, 1996). The same study also suggests that nitric oxide is perhaps part of this CGRP-initiated vasorelaxation process. Taken together, CGRP may induce vasorelaxation with or without involving the endothelium.

SP is also synthesized in the DRG and stored in vesicles in the sensory nerves. SP receptors are located in the endothelium (Maggi, 1995), which means that SP mediates its vasodilatory effect via the endothelium (Marti et al., 1987). Both CGRP and SP sensory fibers are capsaicin-sensitive which are stimulated mechanically, chemically or thermally to release CGRP and SP (Holzer, 1988; Holzer and Maggi, 1998). Although release of CGRP from sensory nerves induces vasodilation in MA and MV, SP plays a relaxing role in MA while it increases venous tone in hypertensive rats (Galligan et al., 2006).

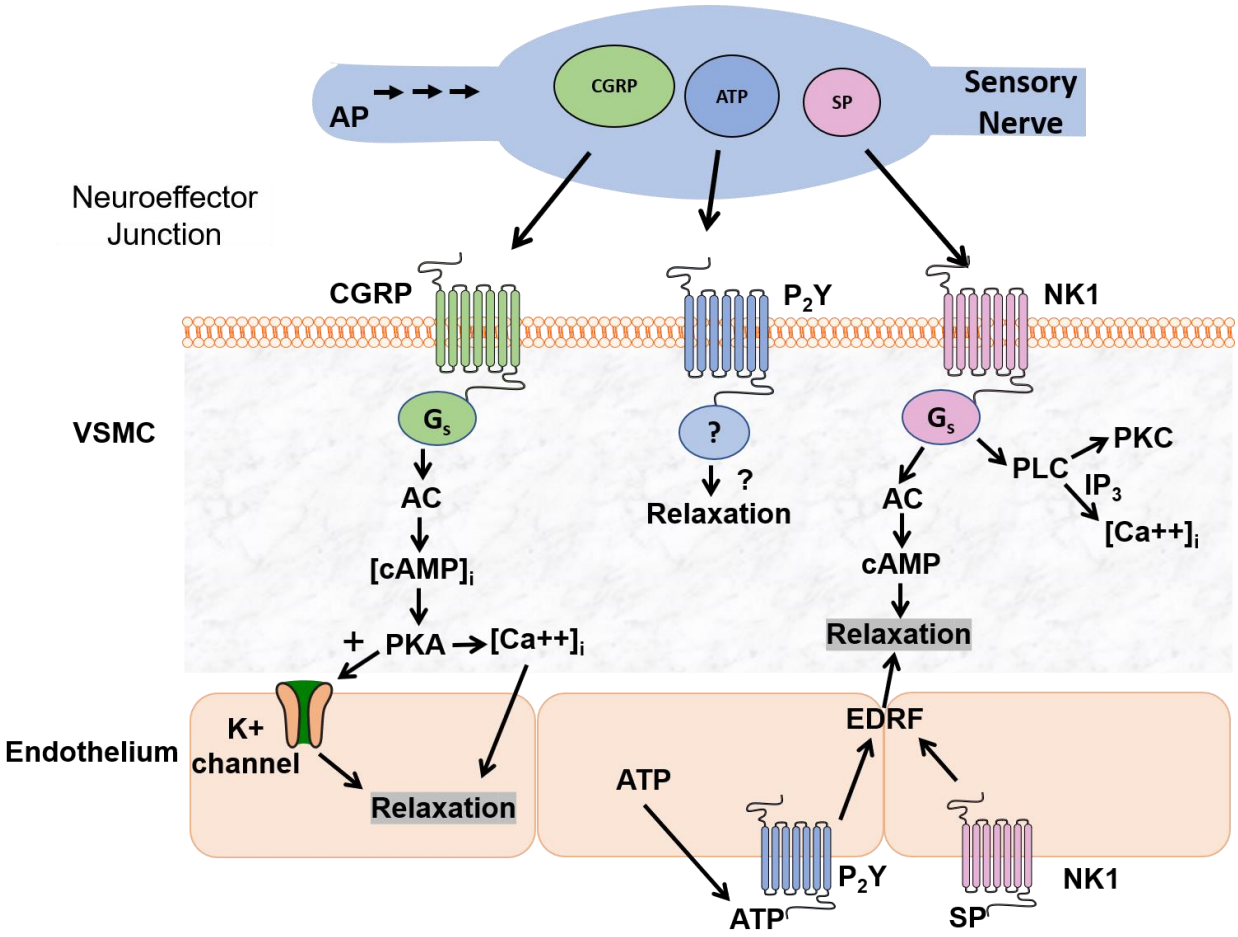


Figure 12. Schematic diagram showing sensory nerve transmission in the vasculature. Calcitonin gene-related peptide (CGRP), ATP and substance P (SP) are released from sensory neurons. CGRP activates adenyl cyclase (AC) that increases the level of cAMP, which in turn induces K^+ efflux from the endothelium through activation of phosphokinase A (PKA). The ATP-induced vasodilation is endothelium-dependent, and releases endothelium-derived relaxing factor (EDRF) into the VSMC. SP-associated vasorelaxation is both endothelium- and VSMC-dependent, and vasorelaxation is EDRF and cAMP activated, respectively.

4.7 Influence of Sex Hormones on Sympathetic Neurotransmission and Blood Pressure

The impact of sex hormones on the sympathetic nervous system and hypertension have been extensively explored. It is widely accepted that estrogen is vasoprotective (Xing et al., 2009), and this was demonstrated by increased blood pressure in ovariectomized rats (Martin et al., 2008). In addition, hormone replacement therapy lowers blood pressure and cardiovascular risk in most cases (Lloyd et al., 2000). Estrogen has genomic and non-genomic mechanisms that lead to vasodilation (Table 4) (Orshal and Khalil, 2004) . Estrogen has α and β receptors on vascular smooth muscle cells (Hogg et al., 2012). They also express a GPCR known as GPR30 that is found on the surface of smooth muscle cells as well (Holm and Nilsson, 2013). In addition, estrogen increases the production of NO in the endothelium to facilitate vasodilation (Guo et al., 2005). These are the mechanisms by which estrogen protects females against increased vascular resistance, and hypertension. This protection is no longer provided for women who are postmenopausal confirming the anti-hypertensive property of estrogen (Staessen et al., 1989; Staessen et al., 1997).

In contrast, testosterone has been associated with increased in blood pressure (Masubuchi et al., 1982; Rowland and Fregly, 1992; Reckelhoff et al., 1998). One suggested mechanism is that testosterone increases reabsorption of sodium, and glomerulosclerosis (Liu and Ely, 2011). Testosterone may also activate the RAAS in young males, which in turn increases blood pressure (Dalmasso et al., 2017). Increase in renal NE release due to testosterone can also be attributed to higher sympathetic activity in male rats (Jones et al., 1998). Moreover, testosterone may also increase

circulating homocysteine which is known to damage blood vessels resulting in atherosclerosis (Giltay et al., 1998). Nevertheless, there are few studies that indicate some benefit from testosterone replacement therapy which improved heart rate variability in men (Poliwczak et al., 2013). Others have shown lower level of testosterone in hypertensive men (Hughes et al., 1989; Phillips et al., 1993; Jaffe et al., 1996), and in men with cardiovascular disease (Kalin and Zumoff, 1990; Phillips et al., 1994). However, the lower testosterone may be related to disease induced stress and there is no cause-effect relationship here. Testosterone also has a vasorelaxant effect on the VSMC and endothelium (Table 4) (Lopes et al., 2012).

Similar to estrogen and testosterone, progesterone lowers blood pressure. This has been shown in men and postmenopausal women following oral administration of progesterone (Rylance et al., 1985). Its mechanism of action involves reduction of plasma calcium ions which results in lower contractility in the blood vessels and the cardiac cells (Table 4) (Dharwadkar et al., 2017). Progesterone has also been shown to amplify the vasodilatory effect of estrogen in dog coronary arteries (Miller and Vanhoutte, 1991). However, in some studies progesterone does not exert any effect (Tapanainen et al., 1989; Barbagallo et al., 2001). Interestingly, synthetically-prepared progesterone for hormonal replacement therapy results in elevated blood pressure (Rosenthal and Oparil, 2000) as a result of renal sodium retention (Oelkers et al., 1974). This could be due to formulation of the synthetic progestins, the impurities, vehicles used or the route of administration.

Table 4. List of sex hormones involved with blood pressure. Shows the different types for estrogen, testosterone, and progesterone receptors that play vasodilatory, vasoprotective, and antihypertension role.

Receptors	Receptor Localization	Tissue	Mechanism	Effect
For estrogen				
ERα and ERβ	Intracellular/cytoplasmic	VSMC and Endothelium	Genomic vasodilation-increase in the expression of NOs	Vasodilation
GPER	Cell membrane	VSMC and Endothelium	Non-genomic vasodilation-facilitates the synthesis of NO	Vasodilation
For testosterone				
BKca channel	Cell membrane	VSMC	K^+ efflux	Vasodilation
AR	Intracellular	VSMC	Causes NADPH oxidase-related ROS production that binds to NO	Reduced vasodilation
	Intracellular	Endothelium	Converted to estrogen by aromatase	vasodilation
For progesterone				
PR	Intracellular	Lung	Decreases plasma Ca^{++} by respiratory alkalosis	Vasodilation In VSMC and cardiac muscle cells

CHAPTER 2: RESEARCH AIMS AND HYPOTHESES

1.0 Overall Research Goals

The purpose of our study was to evaluate the effects of high fat diet (HFD), sex, and age on sympathetic neurotransmission and blood pressure. HFD-associated obesity is a well-established risk factor for cardiovascular disease, such as hypertension, stroke, heart attack, and type 2 diabetes, kidney damage. Among these, the association between obesity and hypertension in the mesenteric vasculature is not clearly understood. Some studies suggest that obesity and hypertension are linked by increased sympathetic neuronal activity in the vascular bed. The rat models previously used from our lab to suggest such mechanisms in DOCA-salt rat on a high salt diet. This model provides insightful knowledge as to how HFD and high salt contribute to the development of hypertension. However, not all obesity models exhibit hypertension. For example, HFD-fed Sprague Dawley rat and C57bi mice can become HFD-induced obese without becoming hypertensive (Xu et al., 2015). Indeed, this observation represents some obesity in human populations. In the case of the DOCA-salt rat, the association of salt with alpha2-AR damage and inflammation as related to sympathetic overdrive explains increase in blood pressure. It is known that chronic high salt consumption leads to hypertension by altering renal function. Now we know that salt has a damaging effect on the neurotransmitter release mechanism in the prejunctional nerves from mesenteric arteries causing a rise in blood pressure. Therefore, the impact of salt in causing hypertension as demonstrated by the DOCA-salt model reflects clinical relevance to non-obese but hypertensive patients.

In our research, we intended to use an animal model that can represent an ever-growing number of obese human population. One of the most common clinical

diagnosis in obese patients is hypertension. Of course, hypertension by itself is not necessarily a disease. Unfortunately, close to 80% of hypertension is related with obesity, and that hypertension in these population is accompanying by a whole host of cardiovascular disease, and type 2 diabetes. We use Dahl salt-sensitive rat (Dahl ss) to mimic salt-sensitive hypertension in hypertension for two main reasons. First, salt-sensitive hypertension is a component of HFD-induced hypertension, and our group has demonstrated that these rats can gain relatively excessive weight and become hypertensive with greater role of sympathetic nervous system in HFD males than HFD females (Fernandes et al., 2018). Second, previous studies successfully demonstrated HFD-induced hypertension in the Dahl ss rat (Nagae et al., 2009; Spradley et al., 2013). Taking those into consideration, the Dahl ss rat was fed HFD with normal salt (0.3% NaCl and 60% kcal fat) in order to induce HFD-associated weight gain.

In general, though while women become more obese than men, hypertension is more prevalent in men than in women. To find an answer to such paradox, we included females in our study. This not only shows us whether there are sex differences in this rat model, but also it allows us to investigate the underlying mechanisms of obesity-associated hypertension in both sexes. There are several differences between the physiology and pathophysiology of men and women. For instance, the distribution of body fat, the difference in the type and amount of peptide hormones (e.g., leptin) released from the adipocytes, the involvement of the immune system, and activation of RAAS. In our study, we acknowledge these and other concomitant factors to the development of hypertension. Nevertheless, our focus is the sympathetic

neurotransmission in mesenteric blood vessels, and how it is different between the sexes.

We selected three time-point to compare the progress and development of HFD-related hypertension. These time points were 10-, 17-, and 24-wk after diet. The reason for choosing these times was that our hypertension data showed that 10-wk rats were pre-hypertensive whereas at 17-wk the rats began to be hypertensive and continued to be so up to 24-wk and possibly beyond. The reason we chose the 24-wk time point was that the changes in blood pressure were not increasing at higher rate as before although they were more hypertensive than the 17-wk group (Fernandes et al., 2018).

2.0 Overall Hypotheses

We will test the hypothesis that HFD-induced increase in sympathetic neurovascular transduction (neurotransmission) is greater in male than female Dah ss rats in MA. We will also test the hypothesis that HFD-induced increase in sympathetic neurotransmission is greater in male than female Dahl ss rat in MV. Lastly, we will test the hypothesis that there are three distinct populations of sympathetic nerves in the CG, MA, and MV.

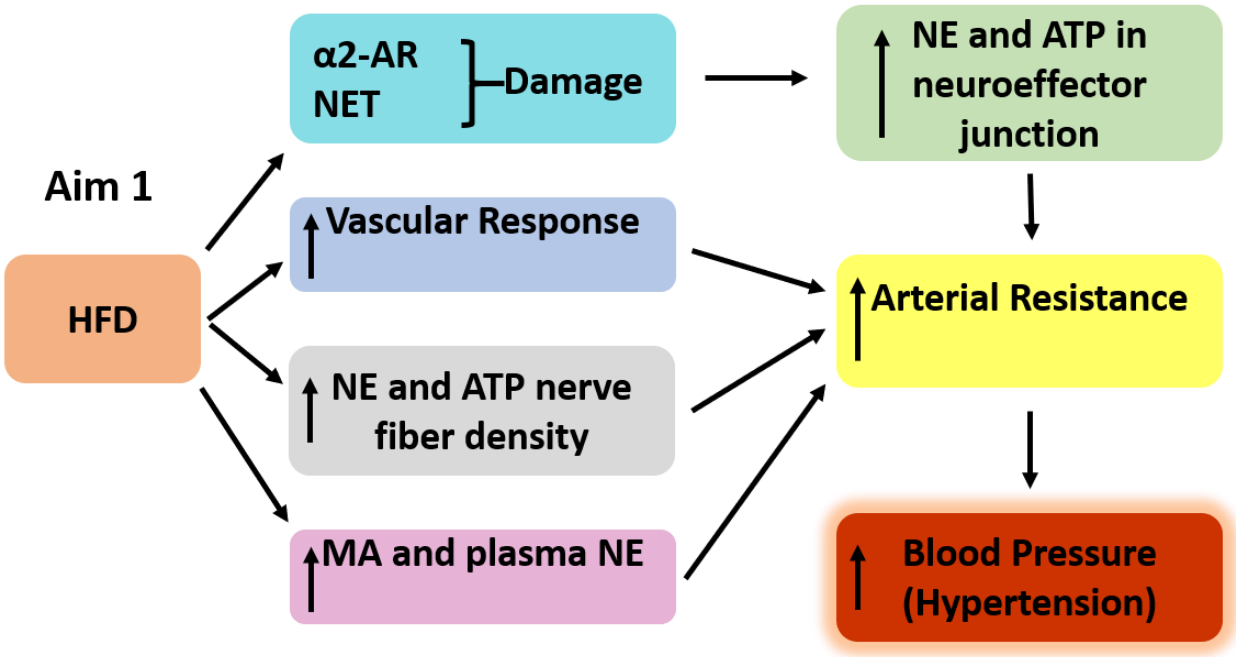


Figure 13. Flow chart for specific Aim 1. A complete story for Aim 1 is shown in Chapter 4 which looks into the effect of HFD on the sympathetic neurotransmission and blood pressure in MA.

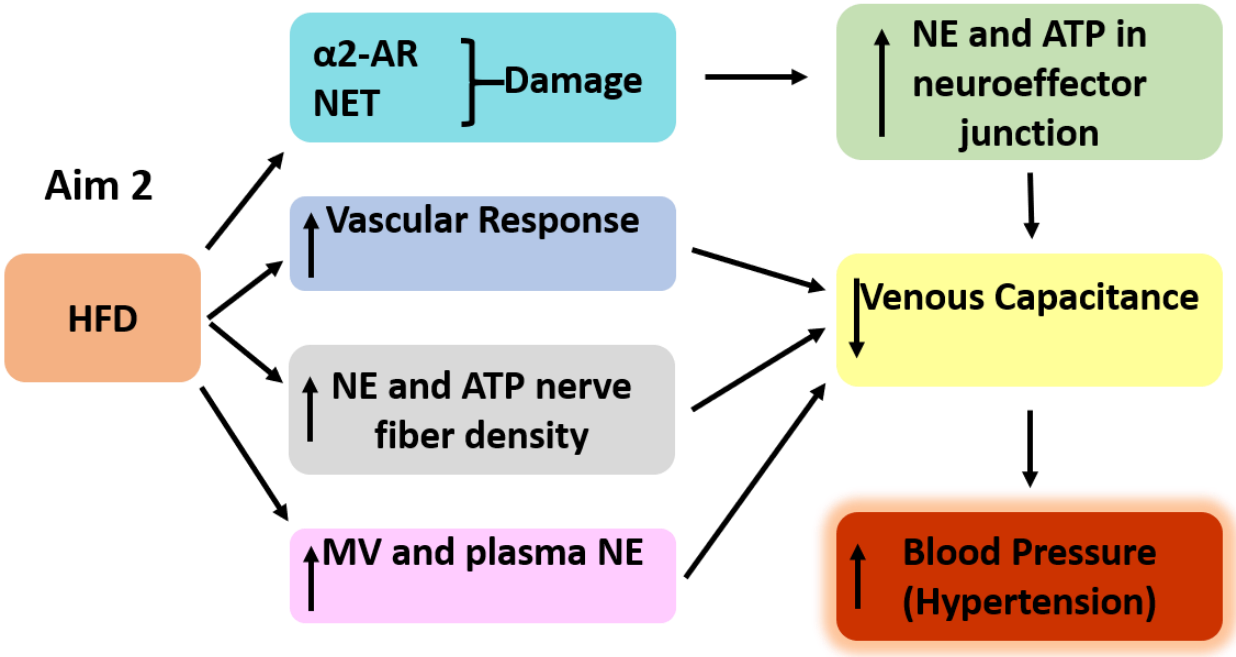


Figure 14. Flow chart for specific Aim 2. A detailed data for Aim 1 is shown in Chapter 5 which investigates the effect of HFD on the sympathetic neurotransmission and blood pressure in MV.

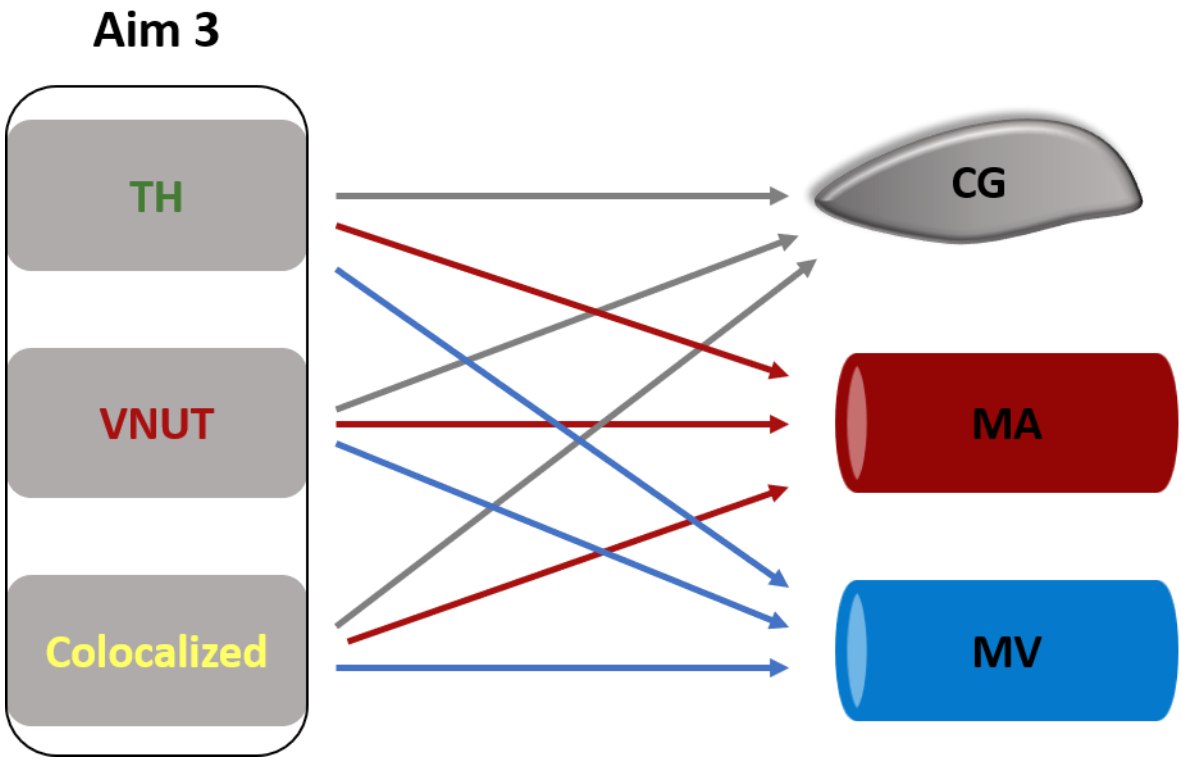


Figure 15. Schematic depiction of specific Aim 3. The detailed data for this section is discussed in Chapter 6 that tests whether there are three distinct populations of sympathetic nerves in CG, MA, and MV.

3.0 Specific Aims

Aim 1: To test the hypothesis that HFD-induced increase in sympathetic neurovascular transduction is greater in male than female in MA from Dahl ss rat (Fig. 12). Nerve stimulation-induced arterial constriction will be used to assess whether HFD induces increase in neurotransmitter release. α2-AR will be activated by UK 14,304 (agonist) to determine if the function of the receptor is impaired or not with HFD feeding. Cocaine will be used to block NE reuptake by the norepinephrine transporter (NET) while stimulating the prejunctional nerves at increasing frequency (0.2 – 30 Hz). Adrenergic and purinergic components of the neurogenic stimulation will be determined by using antagonists against adrenergic and purinergic receptors in the VSMC. TH- and VNUT-ir nerve density will be assessed by immunofluorescence staining and confocal imaging. NE content in MA, and NE level in plasma will be determined by HPLC. Finally, vascular reactivity to exogenous NE and ATP will be assessed.

Aim 2: To test the hypothesis that HFD-induced increase in sympathetic neurovascular transduction is greater in male than female in MV from Dahl ss rat (Fig. 13). Nerve stimulation-induced venous constriction will be used to assess whether HFD induces increase in neurotransmitter release. α2-AR will be activated by UK 14,304 to determine if the function of the receptor is impaired or not with HFD feeding. Cocaine will be used to block NE reuptake by the NET while stimulating the prejunctional nerves at increasing frequency (0.2 – 20 Hz). Adrenergic and purinergic components of the neurogenic stimulation will be determined by using antagonists against adrenergic and purinergic receptors in the VSMC. TH- and VNUT-ir nerve density will be assessed by immunofluorescence staining and confocal imaging. NE

content in MV will be determined by HPLC. Finally, vascular reactivity to exogenous NE and ATP will be assessed.

Aim 3: To test the hypothesis that there is HFD-induced alteration in the distribution of sympathetic nerves in CG, MA, and MV in male and female Dahl ss rat (Fig. 14). Localization of TH and VNUT as well as nerves or vesicles colocalizing both TH and VNUT will be assessed by immunofluorescence staining on the CG. Localization of TH and VNUT as well as nerves or vesicles colocalizing both TH and VNUT will be assessed by immunofluorescence staining on the MA. Localization of TH and VNUT as well as nerves or vesicles colocalizing both TH and VNUT will be assessed by immunofluorescence staining on the MV.

CHAPTER 3: RESEARCH DESIGN AND METHODS

1.0 General Experimental Design

Diet (high fat vs. control), sex (male vs. female) and age (10 vs. 17 Vs. 24 weeks on diet) differences will be assessed in four major parts of the sympathetic neurovascular transmission. First, the state of the prejunctional (presynaptic) control of neurotransmitter release monitored by α2-AR will be assessed with an agonist. Further, the NE reuptake system mediated by NET will be evaluated with an antagonist. Second, vascular reponse to either a direct nerve stimulation or to exogenous neurotrasmitters will be determined by measuring change in vascular inner diameter. Third, the level of NE in tissue and plasma will be determined by HPLC as an indect way of evaluating sympathetic nerve activity. This will be undertaken in conjunction with immunohistochemical analysis of TH and VNUT nerve densities. Fourth, the type of sympathetic nerves in CG, MA, and MV will be explored by using immunohistochemistry, and ImageJ software. Our experimental design is schematically represented in Fig. 15.

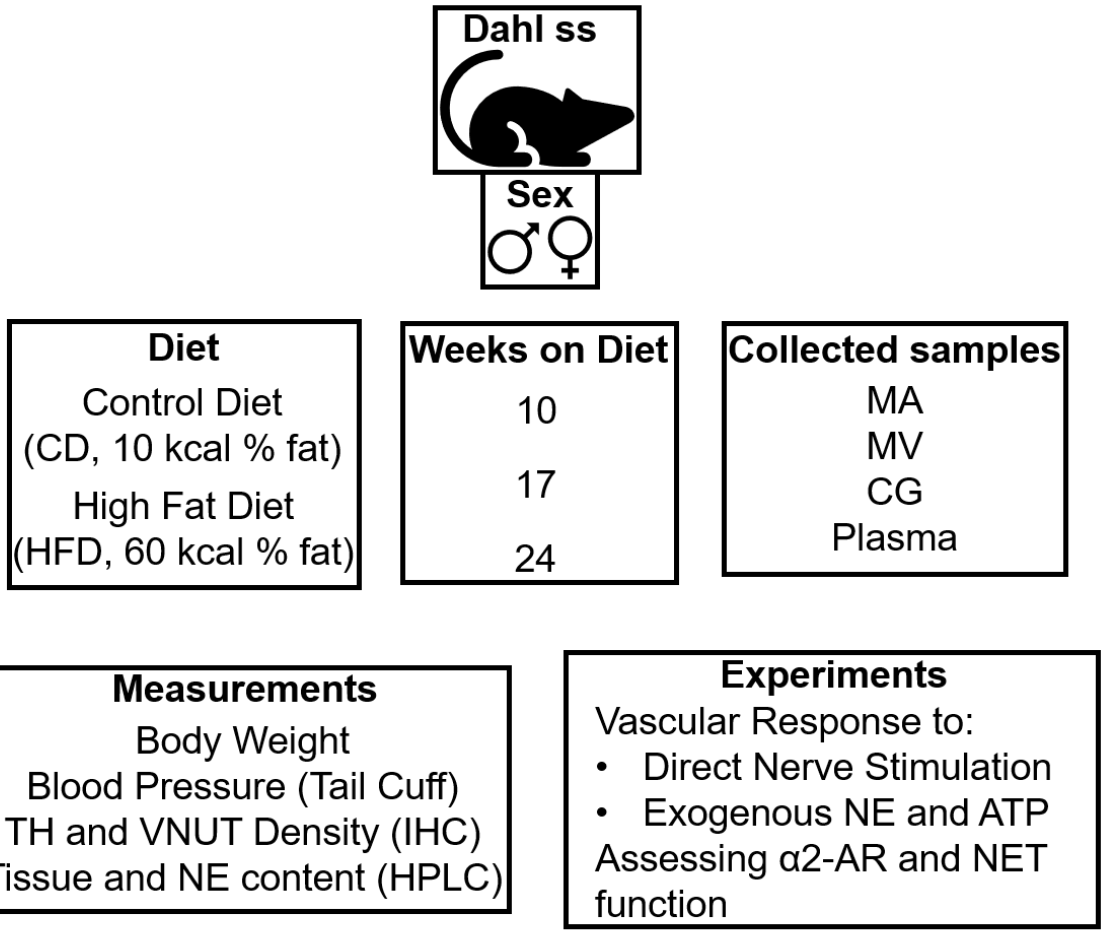


Figure 16. Overview of experimental design. The effect of HFD, age and sex on the sympathetic neurotransmission and on the development of hypertension will be investigated in Dahl ss rat.

2.0 Materials and Methods

Chemicals (CHAPTERS 4 and 5)***.*** NE, ATP disodium salt hydrate, prazosin, and suramin were acquired from Sigma-Aldrich (St. Louis, MO, USA). Tetrodotoxin (TTX) was acquired from Cayman Chemical (Ann Arbor, MI, USA).

Animals (CHAPTERS 4,5, and 6)***.*** All animal use protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University (AUF# 10/17-179-00). Dahl ss rats are genetically salt-sensitive but in our study all rats were fed normal salt diet as we focused on the effects of HFD on blood pressure. The development of hypertension in this rat model results from HFD-induced obesity and its predisposition to salt sensitivity in which obesity-hypertension shares similar characteristics (Nagae et al., 2009).Three week old male and female Dahl ss rats (initial weight=60-75 g, Charles River Laboratories, Portage, MI) were fed either a CD (Research Diets #D12450J: 10 kcal% fat, 0.24% NaCl, 0.36% K^+, 70 kcal% carbohydrate, and 20 kcal% protein) or a HFD (Research Diets #D12492: 60 kcal% fat, 0.33% NaCl, 1% K^+, 20 kcal% carbohydrate, and 20 kcal% protein) with *ad libitum* access to food and tap water. This animal model represents a life-long obesity associated with metabolic disorders. Rats were randomly assigned to either diet. All rats were housed in groups of two or three in transparent plastic cages in a controlled humidity and temperature room with 12:12-h light-dark cycles. Animals were used after 10, 17 or 24-wk on the control or HFD and were fasted for 12 h overnight before euthanasia. These time-points were chosen because telemetrically measured blood pressure in our previous studies began to separate between CD and HFD-fed rats at 10-wk (Pre-hypertensive), and exhibited significant differences at 17-wk (hypertensive)

which continued until 24-wk (more hypertensive) (Fernandes et al., 2018). Rats were euthanized with a lethal dose (4 %) of isoflurane by inhalation. Tail and leg pinch were used to confirm unconsciousness of rats. Tail vein blood samples and MA were harvested from euthanized rats. Blood was collected in heparin-coated tubes and centrifuged at 4000 rpm for 15 minutes at 4 ^{0}C. Then, plasma was collected and stored at -80 ^{0}C until further use.

Body weight and mean arterial pressure (MAP) (CHAPTERS 4, 5, and 6)***.*** Body weight was measured weekly and right before euthanasia. MAP was measured weekly using the tail-cuff plethysmography method (CODA High Throughput, Kent Scientific, CT, USA) as well as by using telemetry (HD-S210, Data Sciences International, St. Paul, MN) which provided us with similar blood pressure readings.

Tissue preparation and video monitoring of blood vessel diameter (CHAPTERS 4 and 5)***.*** Third order blood vessels (inner diameter for MA: male = 275 - 350 µm, female = 245 – 315 µm; inner diameter for MV: male = 350 – 650 µm, female = 350 – 550 µm) from small intestine were isolated from euthanized rats and were pinned flat on a Sylgard®-lined petri dish (Dow Corning, Midland, MI) filled with Krebs solution (in mmol, pH 7.4: 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 11 glucose and 25 $NaHCO_3$) and pressurized (MA: 60 mmHg; MV: 6 mmHg). Surrounding perivascular fat and connective tissue were carefully removed and an arterial segment was transferred to the recording chamber where each end was tied to micropipettes in a pressure myography chamber (Danish Myo Technology, 114P, DMT, Denmark) containing Krebs solution. Myograph allowed the MA to be kept at a near physiological conditions: pressure (MA: 60 mmHg; MV: 6 – 10 mmHg), temperature (37 ^{0}C) and tissue

oxygenation (95% O_2 and 5% CO_2). A video camera was positioned underneath the chamber, and the pressure myograph was connected to a computer. Myoview II software (DMT, Denmark) allowed continuous monitoring of arterial diameter changes in response to drugs or EFS. At the beginning of each experiment, the viability of blood vessel was tested with NE that produced close to 100% constriction.

Transmural EFS of periarterial sympathetic nerves (CHAPTERS 4 and 5)***.*** Mounted mesenteric vessels were positioned in the myograph chamber between two parallel wire electrodes which were connected to a stimulator (Grass Instruments S48, Quincy, MA, USA). Stimulation parameters were 100 V, 0.5 ms pulse duration and 30 stimuli per train with increasing frequency (MA: 0.2 – 30 Hz; MV: 0.2 – 20 Hz). The neurogenic response was validated by applying TTX (3×10^{-7} M) to block the electrically evoked maximum constriction at the end of each experiment. TTX was added to the Krebs solution flowing through the recording chamber.

Prejunctional α2-AR receptor function (CHAPTERS 4 and 5)***.*** The α2-AR agonist, UK 14,304, (10^{-8} – 3×10^{-6} M) was used to inhibit a direct nerve stimulation at 20 Hz for MA and 10 Hz for MV at which a maximum vasoconstriction response is induced. Then, changes in vasoconstriction was measured to assess and compare the function of α2-AR between CD and HFD; male and female; 10-, 17-, and 24-wk rats. The drug was perfused into the myograph chamber with Krebs solution with in-between washes.

NET function (CHAPTERS 4 and 5)***.*** Direct nerve stimulation of increasing frequency was used while perfusing myograph chamber with Cocaine, a NET

antagonist, (10 µM). The effect of Cocaine on the performance of NET was evaluated by measuring changes in vasoconstriction.

Vascular reactivity to exogenous NE and ATP (CHAPTERS 4 and 5)**.** Concentration-response studies were performed by cumulative addition of NE (MA: 10^{-8} – 10^{-5}; MV: 10^{-9} – 10^{-5} M) to flowing Krebs solution to assess adrenergic constriction mediated by α1-ARs in VSMCs. Similarly, concentration-response studies were performed by adding ATP, non-cumulatively, (MA: 10^{-6} – $3x10^{-3}$; MV: 10^{-8} – $3x10^{-3}$ M) to the flowing Krebs solution. This was to determine the purinergic constriction mediated by P2X and P2Y receptors expressed by arterial SMCs. There was a 10 min wash between addition of increasing concentrations of ATP to the Krebs solution. Responses were reported as percentage constriction based on changes from baseline and to the peak agonist-induced constriction normalized to the baseline diameter.

Immunohistochemistry for blood vessels (CHAPTERS 4 and 5)***.*** We used a modified immunostaining procedure for the mesenteric blood vessels as described previously (Mui et al., 2018). Briefly, connective tissue and perivascular fat were carefully removed from third-order blood vessels harvested from euthanized rats. Blood vessels were fixed with Zamboni (4% paraformaldehyde in 0.1 M phosphate buffer (PB) solution, pH 7.4) for 24 h. Tissues were transferred to 70% ethanol and stored at 4 ^{0}C before use. Tissues were washed with 0.1 M PB solution (pH 7.4) and incubated for 10 min in dimethyl sulfoxide (DMSO). Tissues were washed again with 0.1 M PB solution and incubated in blocking solution (0.1 M PB solution, 4% goat serum, 4% donkey serum) for 1 h at room temperature. Tissues were further incubated for 12 h at 4 ^{0}C in blocking solution with primary antibodies targeting TH (1:200; MAB318, Millipore,

Burlington, MA, USA) and the VNUT (1:1000; SC-86312, Santa Cruz Biotechnology, Dallas, TX, USA) to localize sympathetic nerve fibers. Tissues were washed with 0.1 M PB solution and incubated in blocking solution consisting of secondary antibodies (goat anti-mouse Alexa Fluor 488; 1:1000, Thermo Fisher Scientific, Waltham, MA, USA and donkey anti-rabbit Alexa Fluor 594; 1:1000, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature in a covered chamber to prevent photobleaching. Tissues were washed with 0.1 M PB solution and with de-ionized water before mounting them with a mounting media (B0730, Vector Laboratories, Burlingame, CA, USA).

Immunohistochemistry for celiac ganglion (CG) (CHAPTER 6). CG was harvested into Zamboni and fixed for 24 h at 4 ^{0}C. Tissue transferred to 50% ethanol followed by paraffin embedding and sectioning (5 µm thick). Tissue was dewaxed and antigen retrieved using unmasking solution (Vector Laboratories, Burlingame, CA). Then, tissue was stained in the same manner as described above.

Confocal imaging (CHAPTERS 4, 5, and 6). Confocal Z-series images were acquired with 40X objective (0.75 N.A.) for MA and MV, and then combined. 60X objective (1.40 oil) with Nyquist 2.0 zoom-in was used for capturing a section of an image from CG tissue making the final magnification to be 120X. Images were acquired using C2 laser scanning microscope and NIS-Elements software (Nikon Instruments, Melville, NY, USA).

Nerve fiber (varicosity) counting (CHAPTERS 4 and 5)**.** Five images were randomly taken from different regions of each artery and vein. Therefore, the sample size reported for the nerve density data is the total images taken, and not the number of animals used. ImageJ was used to lay a grid over each image of the same area (1262 X

1262 pixels). Then, nerves that cross the vertical and horizontal lines were counted and totaled. This total value is reported here. Image contrast and brightness was adjusted to the same level within MA, and MV using Adobe Photoshop CS6 (300 dpi). The original (default) grid size was used for counting TH- and VNUT-ir nerves that cross vertical and horizontal lines for nerves from MA and MV. The sum of vertical and horizontal crossings is reported here. The “n” value for TH- and VNUT-ir counts represent numbers of images acquired, and not the number of animals used.

Vesicular counting (CHAPTER 6)**.** Five random images from one animal were cropped and resized (3.2 w x 3.2 h, 300 dpi; Adobe Photoshop) as well as contrast adjusted to similar level as MA and MV. CG individual images were opened with ImageJ, and a grid was laid over the image. The size of the grid was increased to view nine regions in CG images. TH, VNUT, and colocalization were counted from each region using a Cell Counter plug-in in imageJ. The “n” value represents the number of varicosities representing TH (green), VNUT (VNUT), and colocalized (yellow).

Tissue and plasma NE content (CHAPTERS 4 and 5)***.*** High-pressure liquid chromatography (HPLC) was used to measure NE level in blood vessels and plasma from tail vein (under brief isoflurane anesthesia) using protocols described previously (Ayala-Lopez et al., 2015). Briefly, MA were weighed, homogenized in 0.1 M perchloric acid in 4 to 1 ratio and centrifuged at 15, 000 g for 10 minutes. The supernatant was removed into a new tube and analyzed by HPLC. The HPLC electrochemical detector potential was set at -300 mV. Plasma samples were mixed with activated aluminum oxide solution that bonds to NE at 8.1 pH of Tris-EDTA. After repeated washes to remove contaminants, 0.2 M acetic acid was added to recover NE.

Data and statistical analysis (CHAPTERS 4, 5 and 6)***.*** Frequency and concentration response graphs were curve-fitted. Data are presented as mean ± SEM. Statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Two-way ANOVA with Sidak's multiple comparison post hoc test was performed when comparing CD- and HFD-fed rats. One-way ANOVA was used to compare groups on the same diet but at different age. *, #, &, and *` $P < 0.05$ was considered statistically significant. Outlier test was conducted using online graphPad tool (https://www.graphpad.com/quickcalcs/Grubbs1.cfm) whenever data points are varied resulting with large error bars.

3.0 Research Ethics for Animal Use

There are two main reasons to justify the use of animals to test our hypotheses: 1) there is no computer modelling or other alternative mechanisms that would allow us to capture the physiological properties of mesenteric blood vessels, sympathetic nerves that innervate blood vessels, and to measure the NE content from tissue and blood; 2) rats in general and Dahl ss rats in particular mimic obesity and obesity-associated hypertension in humans.

Proper animal care is provided for these rats when they are housed in our facility (University facility for animal use, Michigan State University) in compliance with the guidelines of the Institutional Animal Care and Use Committee. Animals are humanely anesthetized before use.

CHAPTER 4: EFFECTS OF HIGH FAT DIET ON SYMPATHETIC NEUROTRANSMISSION IN MESENTERIC ARTERY FROM DAHL SALT-SENSITIVE RAT

1.0 Abstract

Obesity-induced hypertension is driven partly by increased sympathetic neurotransmission. We tested the hypothesis that high-fat diet (HFD)-induced hypertension is driven by increased sympathetic neurotransmission in male but not female mesenteric artery (MA) from Dahl salt-sensitive (Dahl ss) rat. Rats were fed CD (10 kcal % from fat) or HFD (60 kcal % from fat) beginning at 3 weeks (wk) of age and measurements were made at 10-, 17- and 24-wk. Body weight increased with HFD, age and sex. Mean arterial pressure (MAP) was higher in HFD versus CD rats from both sexes at 17- and 24-wk. MA constriction measured using pressure myography, and electrical field stimulation (EFS, 0.2 - 30 Hz) response was greater in HFD versus CD in males at 17-wk (Emax). UK 14,304 (α2-AR agonist; 10^{-8} - $3x10^{-6}$ M) and Cocaine (norepinephrine transporter (NET) antagonist; 10^{-5} M) confirmed that α2-AR and NET functioned properly in HFD-fed rats. Prazosin (α1-AR antagonist; 0.1 μM) and suramin (P2 receptor antagonist; 100 μM) inhibited neurogenic MA constriction but did not change the proportion of constriction caused by adrenergic and purinergic stimulation. Arterial reactivity to exogenous norepinephrine (NE; 10^{-8} - 10^{-5} M) was lower in HFD versus CD at 10-wk in males. Arterial reactivity to exogenous ATP (10^{-6} - $3x10^{-3}$ M) was lower in HFD versus CD at 24-wk in females. HFD did not affect Tyrosine hydroxylase (TH) and Vesicular nucleotide transporter (VNUT) nerve density in both sexes. NE content was lower in MA but higher in plasma at 24-wk compared to 10- and 17-wk in both sexes. In conclusion, HFD-induced hypertension is not driven by sympathetic neurotransmission increase in MA in male and female Dahl ss rats.

2.0 Introduction

Obesity is a risk factor for hypertension (Hall et al., 2015) and increased sympathetic nervous system (SNS) activity can contribute to obesity-associated hypertension (da Silva et al., 2009). Mechanisms responsible for SNS effects on obesity-associated hypertension include impaired regulation of sympathetic nerve activity(Esler et al., 2006; da Silva et al., 2009), increased vascular reactivity to sympathetic neurotransmitters (Sivitz et al., 2007; Jerez et al., 2012) and increased sympathetic neurotransmission (Julius et al., 2000; Kalil and Haynes, 2012). Obese Sprague Dawley (Haddock and Hill, 2011; Mui et al., 2018), DOCA-salt (Luo et al., 2004; Park et al., 2010; Kandlikar and Fink, 2011), and spontaneously hypertensive (SHR) rats (Judy et al., 1976; Bencze et al., 2016) are commonly used to study sympathetic neurotransmission in hypertension. Similarly, Dahl ss rats are widely used model for studies of salt-sensitive hypertension (Gillis et al., 2015; Takahashi et al., 2017), and recently few studies have reported on the effect of a high-fat diet (HFD) on blood pressure in this rat model (Zhang et al., 1999; Nagae et al., 2009; Fernandes et al., 2018). However, there have been no studies of sympathetic neurotransmission in resistance arteries (i.e., “neurovascular coupling”) from Dahl ss rats.

Sympathetic nerves are important in regulating vascular tone in the mesenteric blood vessels as studies have proved it by celiac ganglionectomy (King et al., 2007; Li et al., 2010) and ganglionic blockade (Fink et al., 2000). Sympathetic neurotransmission in MAs is mainly mediated by NE and ATP which are co-released from periarterial sympathetic nerve varicosities. Transmitter binding to adrenergic and purinergic receptors causes arterial constriction (Townsend et al., 2016). NE and ATP release is

regulated through negative feedback inhibition by prejunctional α2-AR. Impaired α2-AR in DOCA-salt rats is known to increase NE and ATP release from sympathetic nerves (Park et al., 2010) resulting in a greater vasoconstriction. Moreover, the availability of NE in the neuroeffector junction depends on its clearance by the NET located at the sympathetic nerve varicosities, and blockade of NET leads to higher vasoconstriction (Park et al., 2006). Nevertheless, the specific impact of HFD on these control mechanisms, and on vascular reactivity, is not well established in Dahl ss hypertensive rats. Furthermore, the effect of age, and sex on α2-AR, NET, nerve fiber density and transmitter level has not been studied in Dahl ss rats.

Sex differences in the impact of SNS activity on obesity-associated hypertension have been studied previously (Faulkner and Belin de Chantemele, 2018), with most results supporting a larger impact in male compared to female rats. While obesity-associated changes in SNS activity and vascular reactivity have been studied extensively, only one published study specifically explored sympathetic neurotransmission in obesity-associated hypertension (Haddock and Hill, 2011) and none have addressed possible sex differences. Recent study from our group has shown that sympathetic nervous system is greatly involved in HFD-fed and hypertensive males than in HFD-fed hypertensive females showing sex difference in sympathetic control in HFD-induced hypertension (Fernandes et al., 2018). Therefore, the goal of the work reported here was to evaluate sympathetic neurotransmission in a animal model of obesity-associated hypertension, with an emphasis on identifying possible sex differences in how obesity affects blood pressure in MA. We studied sympathetic neurotransmission in arteries from the mesentery (neurovascular transduction)

because of the importance of the splanchnic circulation in overall regulation of blood pressure. Specifically, we tested the hypothesis that HFD-induces a greater increase in sympathetic neurotransmission in MA of male compared to female Dahl ss rats.

3.0 Results

Effects of HFD, age, and sex on BW and MAP. Body weight was greater in HFD-fed compared to CD-fed rats at 10-, 17-, and 24-wk in both sexes. It also increased with advancing age in both sexes on CD and HFD. Males gained more weight than females regardless of diet at all time points. Mean arterial pressure was greater in HFD-fed compared to CD-fed rats at 17- and 24-wk in both sexes. It was also higher at 24- *versus* 10-wk in males on HFD. In addition, there was a significant increasing trend in MAP in female rats with age (effect of time: $p<0.0001$) (Table 5).

Table 5. BW and MAP measurements. BW and MAP measures for male and female Dahl ss rats on CD and HFD for 10-, 17-, and 24-wk.

		Male			Female		
		10-wk	**17-wk**	**24-wk**	**10-wk**	**17-wk**	**24-wk**
BW (g)[&]	**CD**	**347** ± 5.3 (n=19)	**409** ± 3.5 (n=32)	**466** ± 5.9 (n=29)	**224** ± 2.5 (n=19)	**250** ± 4.7 (n=20)	**273** ± 4.0 (n=20)
	HFD	**374** ± 6.1***** (n=19)	**458** ± 4.6***** (n=30)	**499** ± 6.5***** (n=32)	**242** ± 3.8***** (n=19)	**272** ± 5.1***** (n=20)	**293** ± 3.9***** (n=20)
MAP (mmHg)	**CD**	**137** ± 2.5[#] (n=21)	**125** ± 4.1 (n=25)	**124** ± 5.6 (n=18)	**122** ± 4.6 (n=20)	**115** ± 4.6 (n=21)	**142** ± 8.1[#] (n=15)
	HFD	**138** ± 4.7 (n=21)	**145** ± 4.0***** (n=26)	**155** ± 4.7*****[#] (n=20)	**121** ± 4.6 (n=18)	**138** ± 5.5*****[#] (n=19)	**162** ± 5.7*****[#] (n=16)

*****Indicates significant difference between CD- and HFD-fed groups. [&]Indicates increase in BW with age irrespective of diet in both sexes. [#]Indicates higher MAP at 17- and 24-wk compared to 10-wk in males on CD; indicates higher MAP at 24- compared to 10-wk in males on HFD; shows higher MAP at 24-wk compared to 10- and 17-wk in females on CD; shows increase in MAP with advancing age in females on HFD.

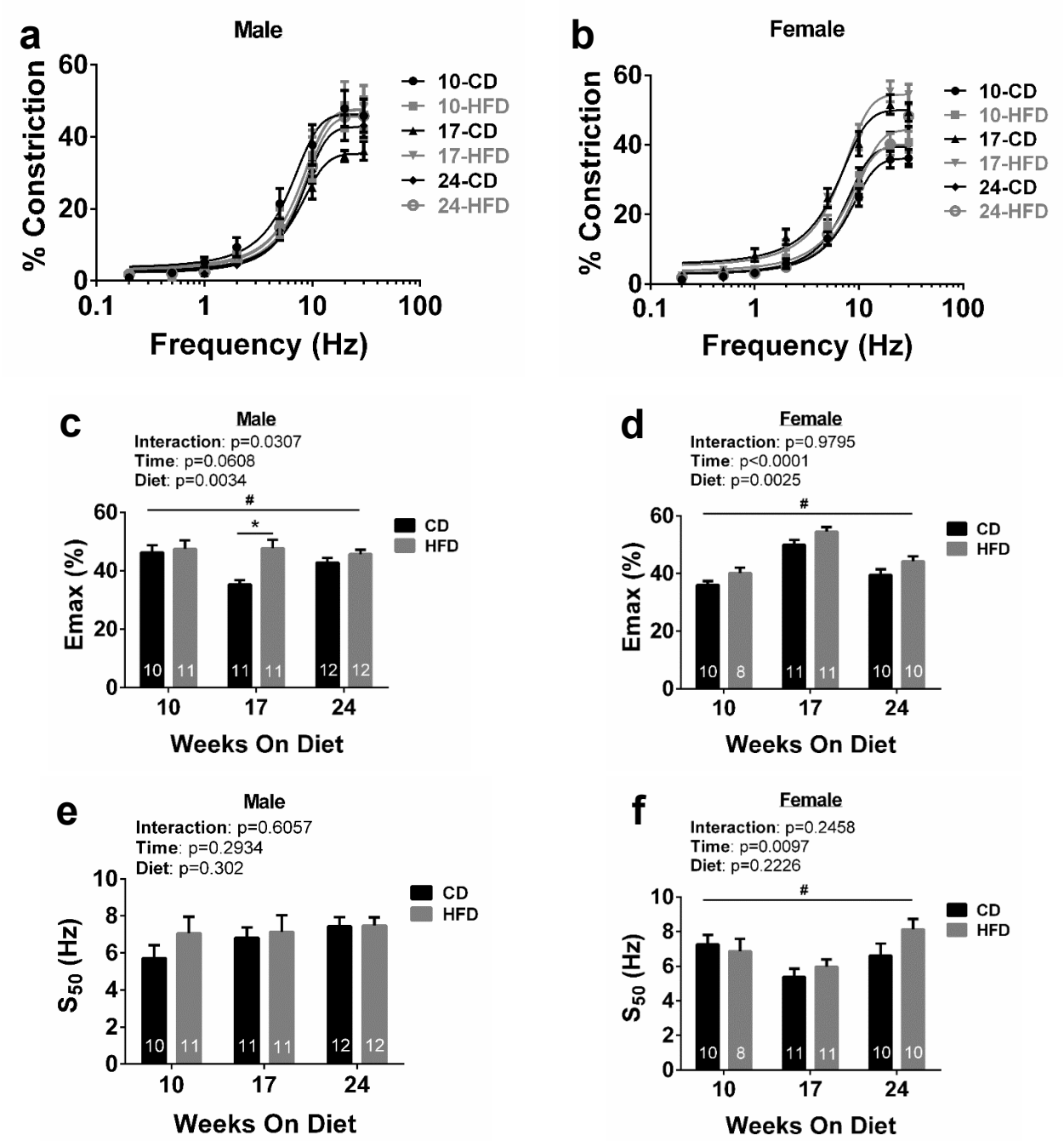


Figure 17. Vasoconstriction in response to direct periarterial nerve stimulation. ***a***: Frequency response curves for male rats. ***b***: Frequency response curves for females. ***c***: Maximum frequency response (Emax) comparing CD and HFD at 10-, 17-, and 24-wk in males. *****Emax is greater in HFD versus CD at 17-wk. #Emax for CD is lower at 17-wk compared to other times. ***d***: Emax comparing CD and HFD at 10-, 17-, and 24-wk in females. #Emax is greater at 17-wk (in both diets) compared to the other times. ***e***: Frequency for half maximum response (S_{50}) comparing CD and HFD at 10-, 17-, and 24-wk in males. ***f***: S_{50} comparing CD and HFD at 10-, 17-, and 24-wk in females. #S_{50} is lower at 17-wk (in both diets) compared to the other times.

***Effects of HFD, age, and sex on nerve-mediated vasoconstriction*.** Curve-fitted log of frequency response curves for EFS (0.2 – 30 Hz) were used to compare nerve-mediated constriction in male (Fig. 17a) and female (Fig. 17b) rats. Neurogenic constriction was greater in HFD- compared to CD-fed rats in 17-wk males but not females (Fig. 17c, d). However, this difference is due to lower Emax in CD-fed males at 17-wk and not necessarily due to increase in Emax in the HFD-fed males at 17-wk. Female (but not male) rats exhibited higher Emax and lower S_{50} at 17-wk compared to 10- and 24-wk, but this was not affected by diet.

***Effect of UK 14304 on neurogenic transmission in MA*.** UK 14304 is an agonist for α2-AR located on the nerve terminal that inhibits NE and ATP release leading to decreased vasoconstriction. Thus, using 20 Hz EFS and UK 14304 (10^{-9} - $3x10^{-6}$ M) we examined whether the function of α2-AR is affected by HFD (Fig. 18a and b). The maximum inhibition (Imax) does not change in males (Fig. 18c); and females (Fig. 18d). The concentration of UK 14304 that produces half-maximum of inhibition (IC_{50}) does not change in males (Fig. 18E); yet, it increases in 17-wk HFD females (Fig. 18f).

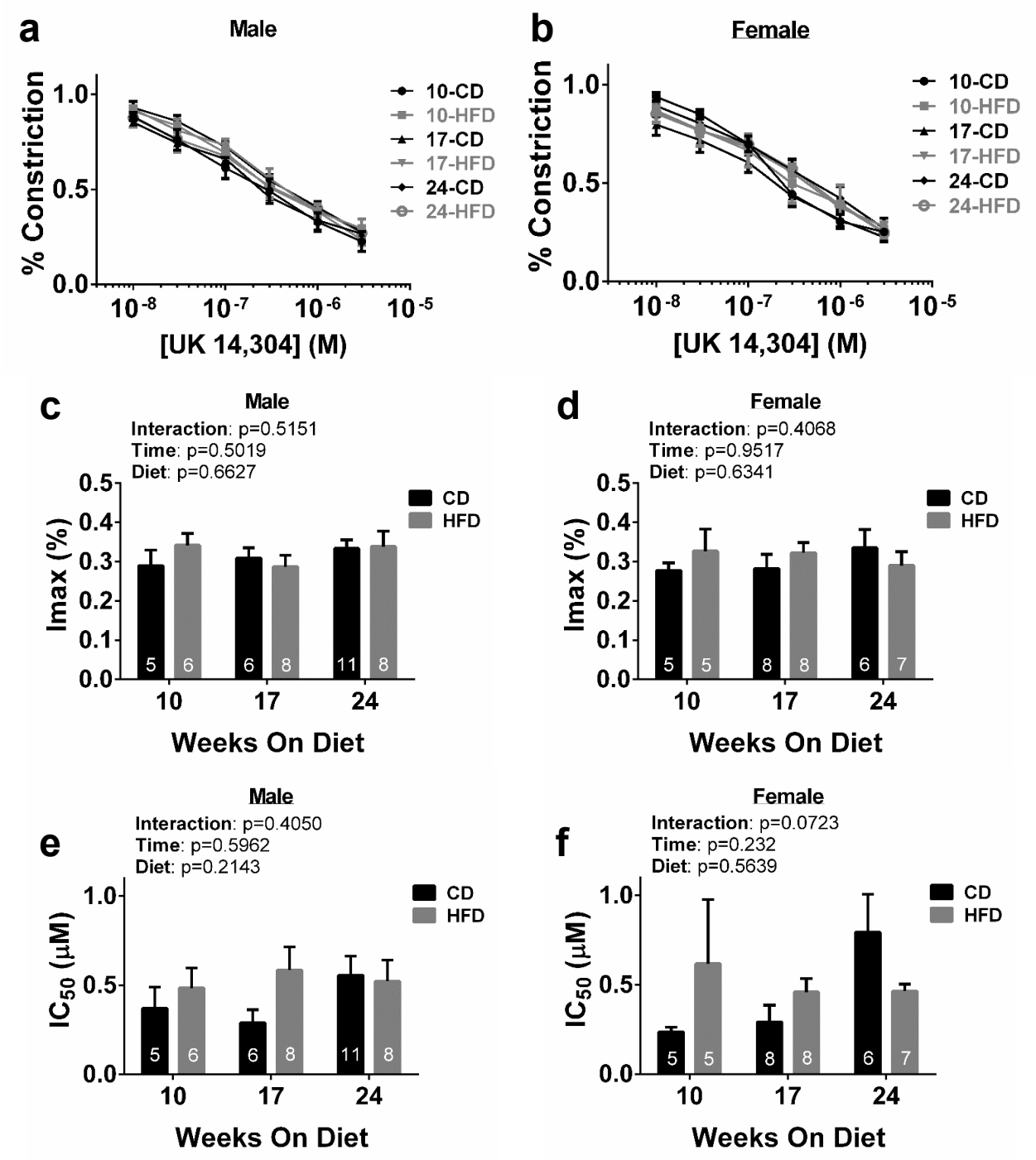


Figure 18. Inhibition of arterial constriction by activated α2-AR. ***a***: % constriction response to increasing concentration of UK 14,304 in male rats. ***b***: % constriction response to increasing concentration of UK 14,304 in female rats. ***c***: Imax for % constriction in response to increasing UK 14,304 in males. ***d***: Imax for % constriction in response to increasing UK 14,304 in females. ***e***: IC_{50} for % constriction in response to increasing UK 14,304 in males. ***f***: IC_{50} for % constriction in response to increasing UK 14,304 in females.

***Effect of Cocaine on neurogenic arterial constriction*.** Cocaine is an antagonist for the NET that leads to increase in vasoconstriction. Therefore, we tested if NET was affected by cocaine with EFS (0.2 - 20 Hz). Perfusion of Cocaine induced greater effect of Emax, and lower S_{50} values in males at all time points (Fig. 19a, c). These indicate that the function of NET was intact in males regardless of diet and age. Cocaine increased vasoconstriction in females (Fig. 19b, d).

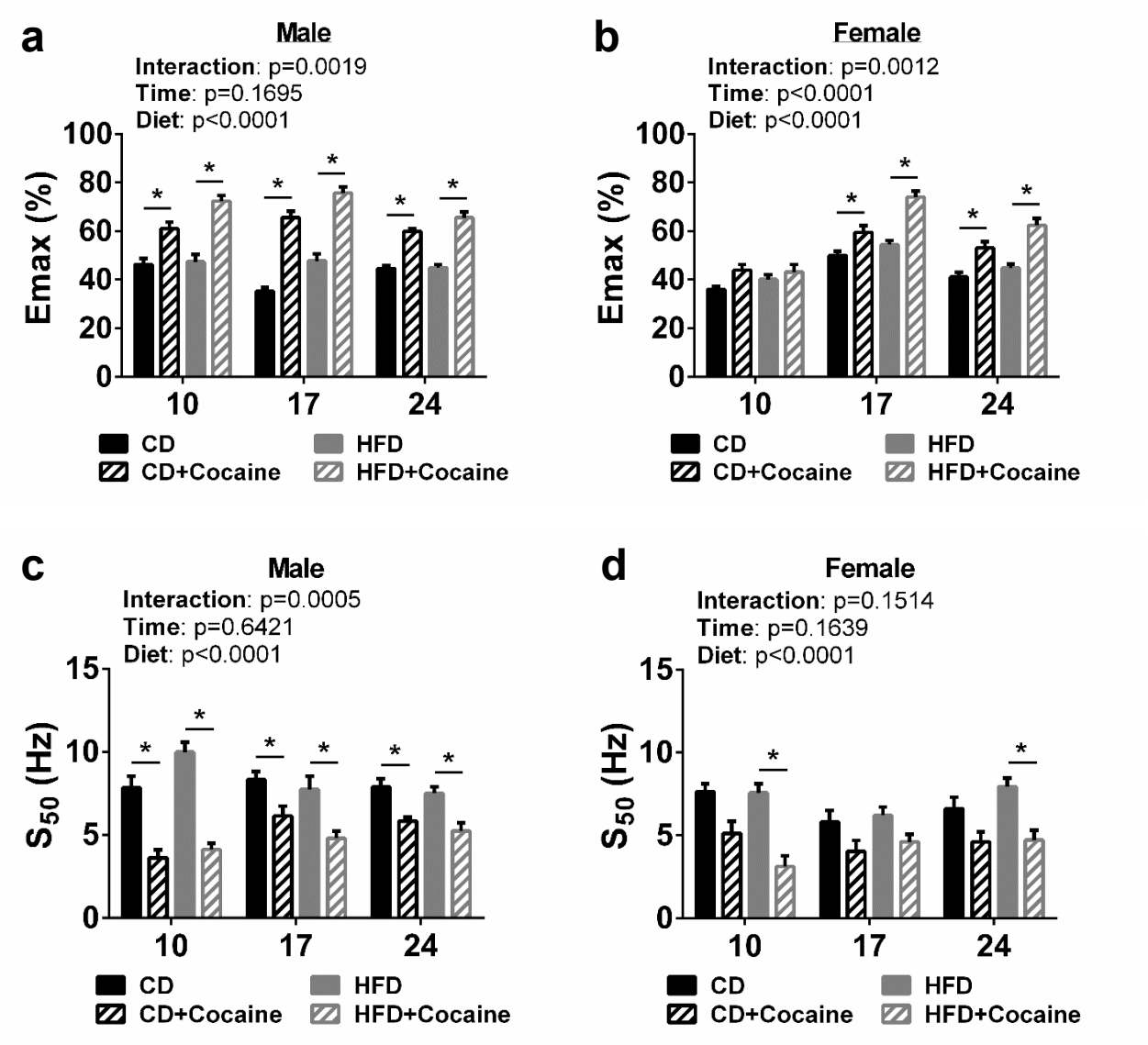


Figure 19. Cocaine-induced change in arterial constriction. ***a***: Emax evaluated in Cocaine perfused and Cocaine free Krebs solution in males. ***b***: Emax evaluated in Cocaine perfused and Cocaine free Krebs solution in females. ***c***: S_{50} for Cocaine perfused and Cocaine free Krebs solution in males. ***d***: S_{50} Cocaine perfused and Cocaine free Krebs solution in females. *****Indicates significant increase in vasoconstriction following cocaine treatment.

***Effects of HFD, age, and sex on the adrenergic and purinergic components of vasoconstriction*.** Adrenergic and purinergic components of neurovascular coupling were determined by comparing constrictions in the presence of respective antagonists (prazosin and suramin) to the baseline response at 20 Hz of EFS (Fig. 20a, b, c). Close to 90% of the constriction was blocked by TTX which confirmed that the constriction was neurogenic (Fig. 20d). The proportion of adrenergic constriction was not different between HFD- and CD-fed rats in both sexes. Furthermore, there was no age- or sex-related difference in adrenergic proportion (Fig. 20e, f). The proportion of purinergic constriction was also similar between HFD- and CD-fed rats in both sexes (Fig. 20g, h).

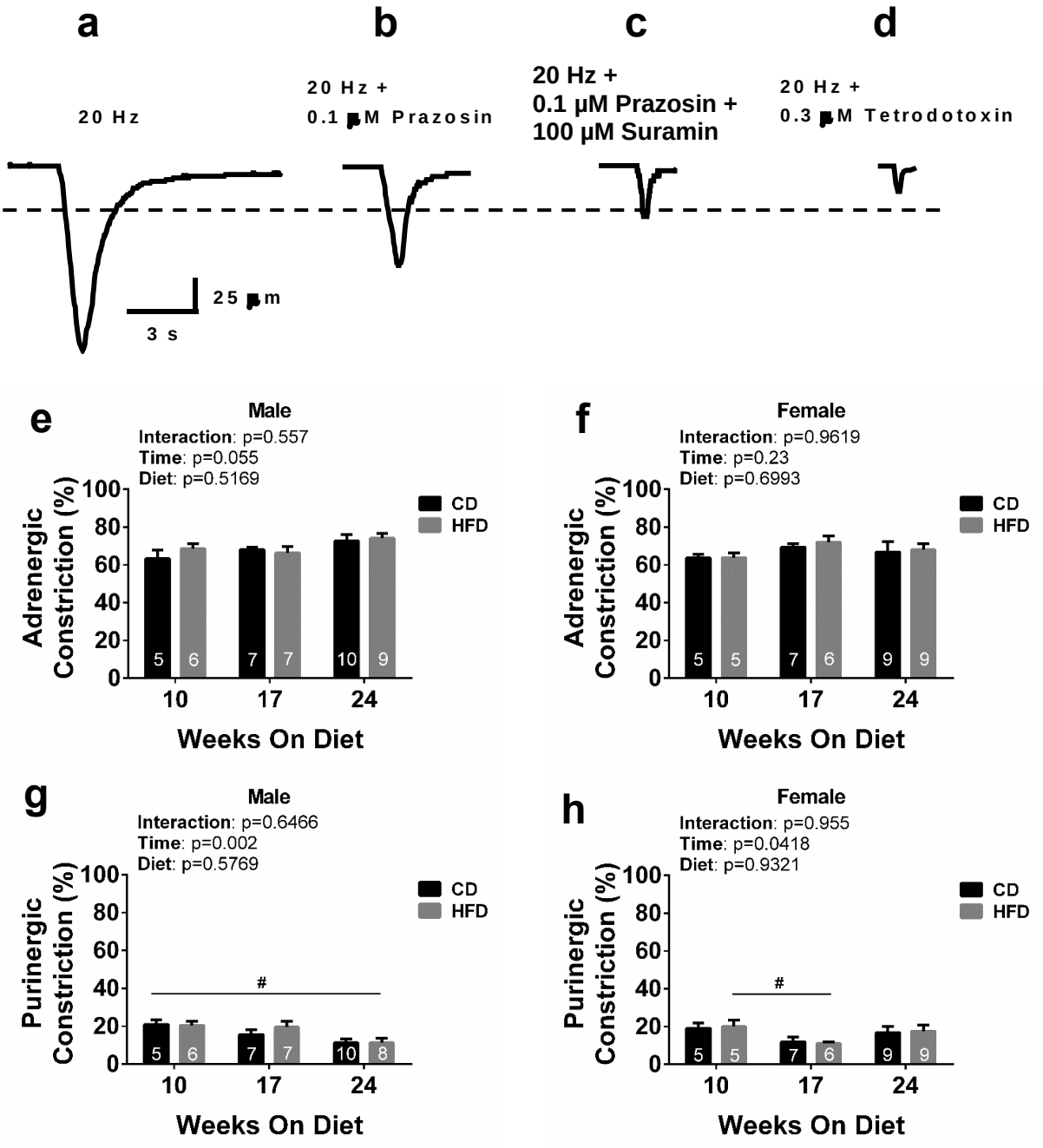


Figure 20. **Proportion of adrenergic and purinergic constriction in MA**. Representative traces of nerve stimulation. ***a***: 10 Hz. ***b***: 10 Hz + 0.1 μM Prazosin. ***c***: 10 Hz + 0.1 μM Prazosin + 100 μM Suramin. ***d***: 10 Hz + 0.3 μM Tetrodotoxin. ***e***: Adrenergic proportion compared between CD and HFD at 10-, 17-, and 24-wk in males. ***f***: Adrenergic proportion compared between CD and HFD at 10-, 17-, and 24-wk in males. ***g***: Purinergic proportion compared between CD and HFD at 10-, 17-, and 24-wk in males. #Purinergic component is lower at 24-wk (in both diets) compared to the other times. ***h***: Purinergic proportion compared between CD and HFD at 10-, 17-, and 24-wk in females. #Purinergic component is lower in HFD at 17-wk compared to HFD at 10-wk.

Effects of HFD, age, and sex on vascular reactivity to exogenous NE and ATP. Concentration-response curves for NE and ATP were plotted to determine the *E*max and EC_{50} in MA from male and female rats (Fig. 21a, b and 22a, b). *E*max was not different between HFD- and CD-fed rats in both sexes (Fig. 21c, d). EC_{50}, on the other hand, was higher in HFD- compared to CD-fed rats at 10-wk in males. EC_{50} was also lower at 17- compared to 10-wk in males on HFD. Nevertheless, it was greater at 24- compared to 10- and 17-wk in both sexes (Fig. 21e, f) indicating a decrease in vascular reactivity to exogenous NE with advancing age. EC_{50} for exogenous ATP was greater in HFD- *versus* CD-fed rats in 24-wk females. Like NE, EC_{50} was greater at 24- compared to 10- and 17-wk in in both sexes (Fig. 22e, f) indicating a decrease in vascular reactivity to exogenous ATP with advancing age.

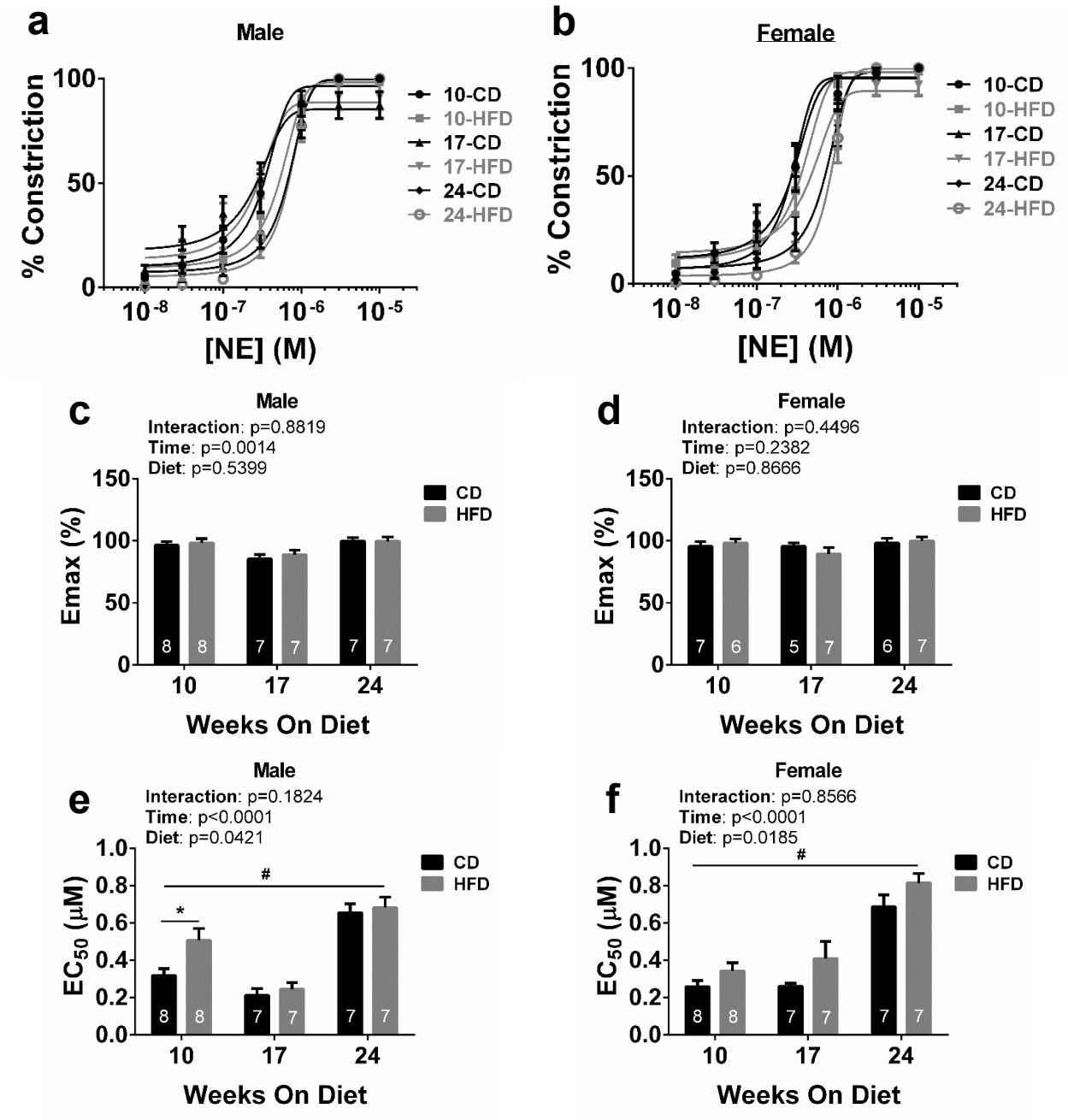


Figure 21. **Arterial reactivity to exogenous NE**. ***a***: NE concentration-response curves for males. ***b***: NE concentration-response curves for females. ***c***: Emax for NE concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in males. ***d***: Emax for NE concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in females. ***e***: EC_{50} for NE concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in males. ***** EC_{50} is greater in HFD than CD at 10-wk. $^{\#}EC_{50}$ is lower in HFD at 17-wk compared to HFD at 10-, and 24-wk. ***f***: EC_{50} for NE concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in females. $^{\#}EC_{50}$ in HFD is greater at 24-wk than HFD in the other times.

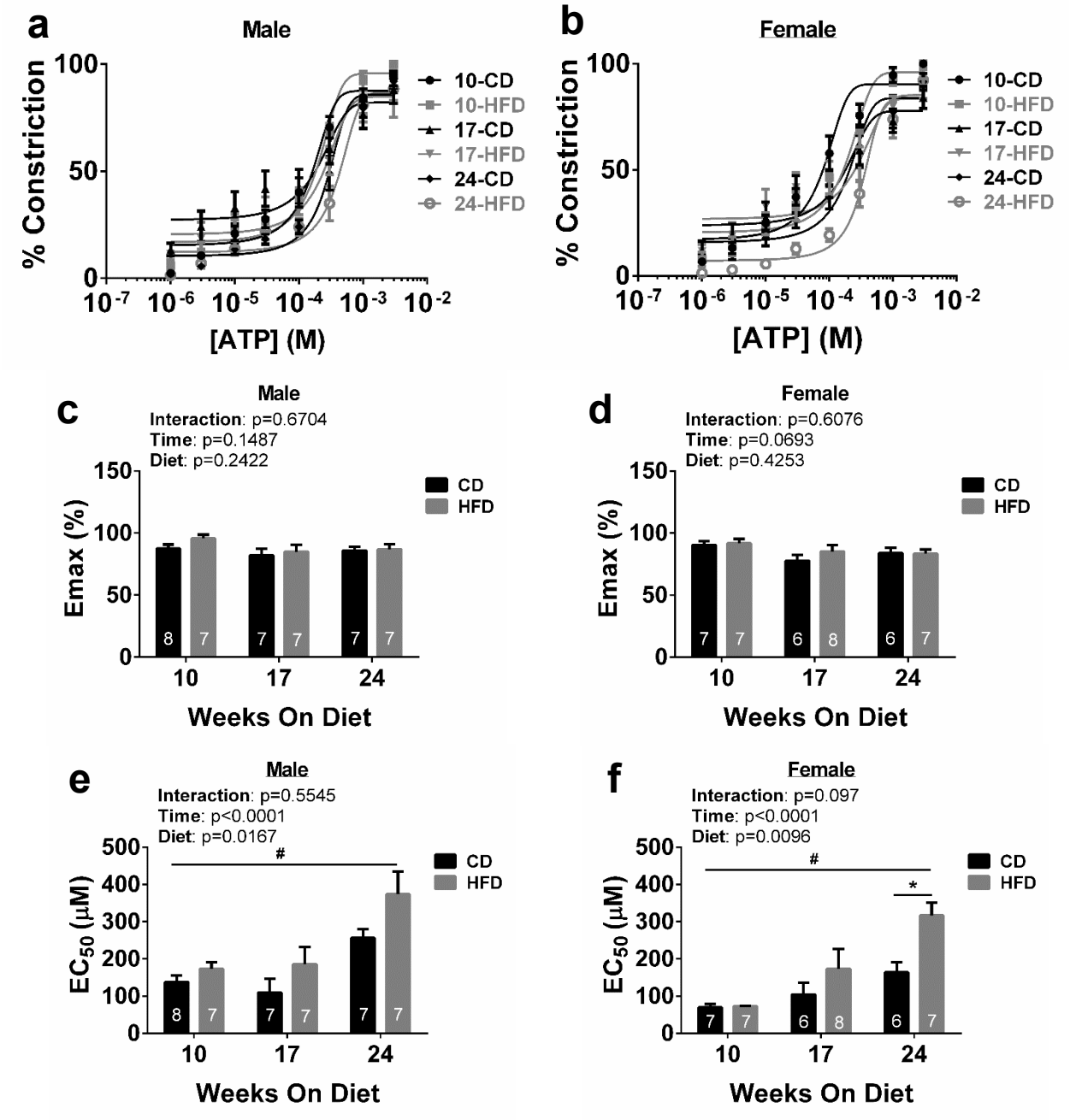


Figure 22. **Arterial reactivity to exogenous ATP**. ***a***: ATP concentration-response curves for males. ***b***: ATP concentration-response curves for females. ***c***: Emax for ATP concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in males. ***d***: Emax for ATP concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in females. ***e***: EC_{50} for ATP concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in males. $^{\#}EC_{50}$ at 24-wk (in both diets) is greater than in the other times. ***f***: EC_{50} for ATP concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in females. $^{*}EC_{50}$ is greater in HFD than CD at 24-wk. $^{\#}EC_{50}$ in HFD at 24-wk higher than HFD in the other times.

***Effects of HFD, age, and sex on TH nerve density*.** The number of TH-ir nerve fibers was counted in MA using confocal microscopy (Fig. 23a, b). There is no difference in TH nerve density between HFD and CD (Fig. 23c) in males. Similarly, the is no difference in TH nerve density between HFD and CD in females. However, TH nerve density was greater in CD at 24-wk compared to CD at 10-wk in females (Fig. 23d).

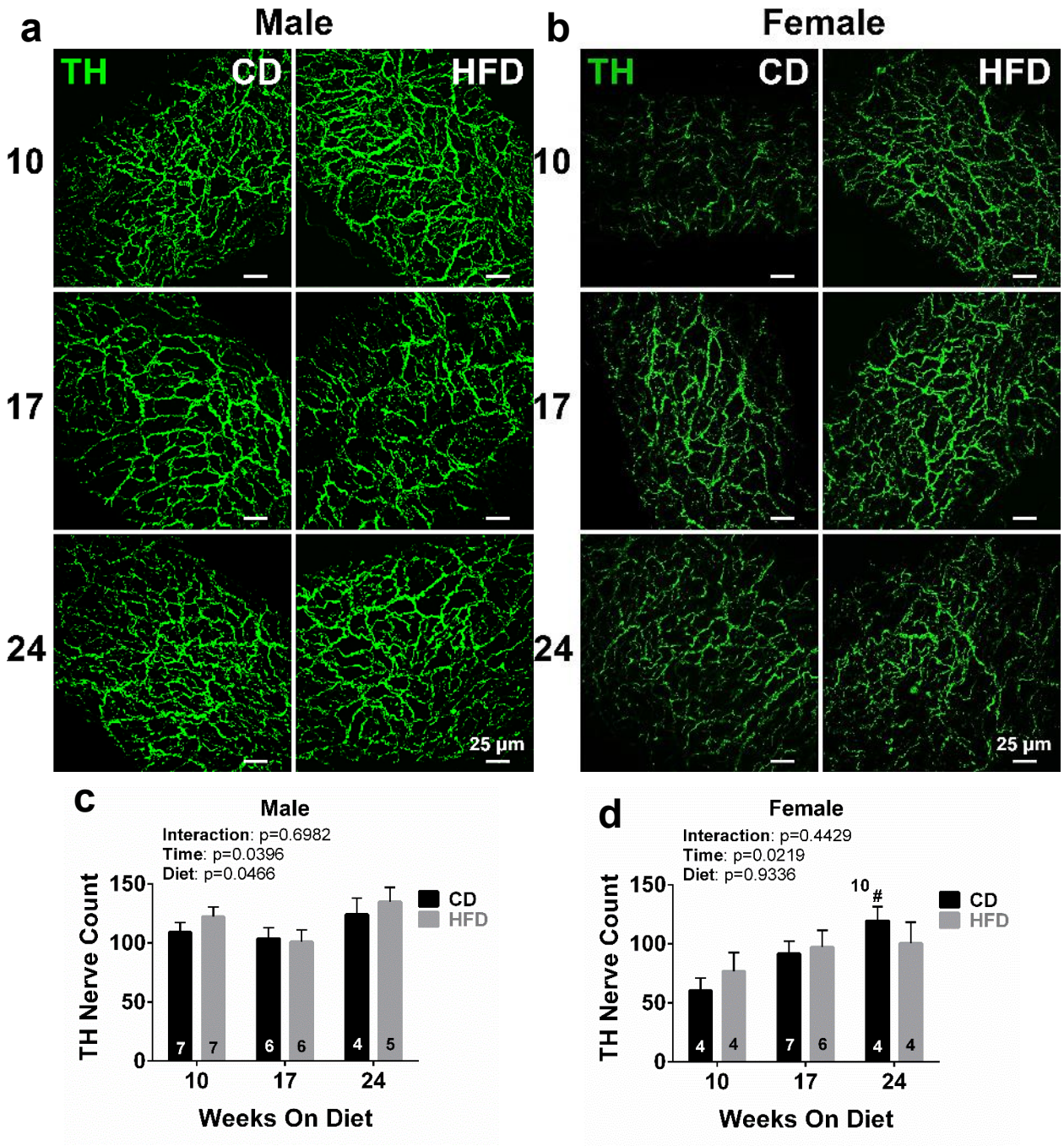


Figure 23. **Periarterial TH nerve density.** ***a***: Representative TH-ir nerve fiber immunofluorescent confocal images in male rats. ***b***: Representative TH-ir nerve fiber immunofluorescent confocal images in female rats. ***c***: TH-ir nerve fiber density shown in CD and HFD at 10-, 17-, and 24-wk in males. ***d***: TH-ir nerve fiber density shown in CD and HFD at 10-, 17-, and 24-wk in females. #TH-ir nerve density is greater in CD at 24-wk compared to CD at 10-wk.

***Effect of HFD, age, and sex on VNUT nerve density*.** VNUT-ir nerves were imaged and counted to assess nerve density in MA (Fig. 24a, b). VNUT nerve count was greater at 24-wk compared to 17-wk in HFD-fed male rats (Fig. 24c). In female rats, VNUT-ir nerve density is higher at 24-wk compared to 10- and 17-wk in CD. Moreover, VNUT-ir nerve density is greater at 24-wk compared to 17-wk in HFD (Fig. 24d).

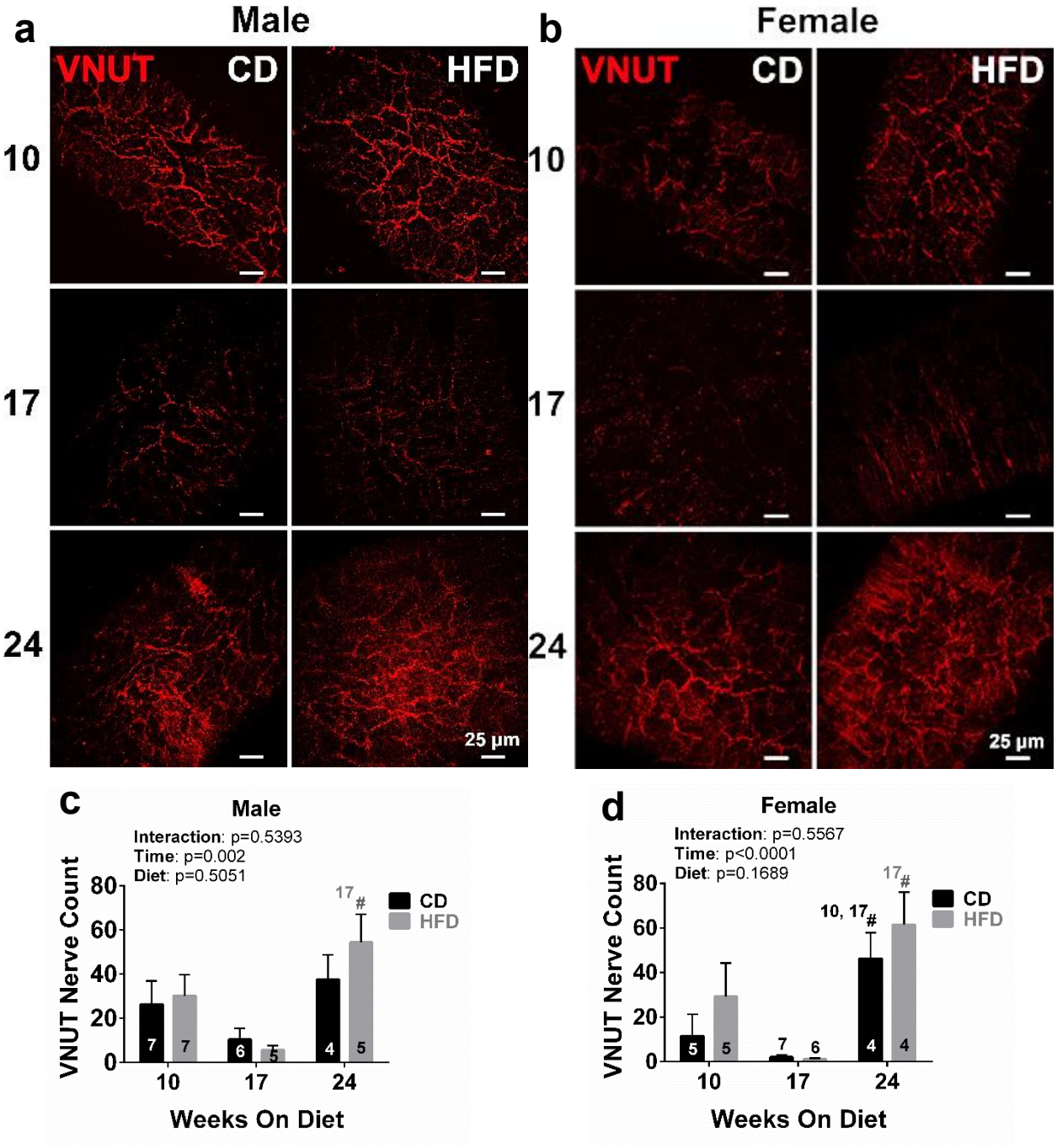


Figure 24. **Periarterial VNUT nerve density.** ***a***: Representative VNUT-ir nerve fiber immunofluorescent confocal images in male rats. ***b***: Representative VNUT-ir nerve fiber immunofluorescent confocal images in female rats. ***c***: VNUT-ir nerve fiber density shown in CD and HFD at 10-, 17-, and 24-wk in males. # (grey)VNUT-ir nerve density is lower in HFD at 17-wk compared to HFD at 24-wk in males. ***d***: VNUT-ir nerve fiber density shown in CD and HFD at 10-, 17-, and 24-wk in females. # (black)VNUT-ir nerve density is higher at 24-wk compared to 10- and 17-wk in CD. # (grey)VNUT-ir nerve density is greater at 24-wk compared to 17-wk in HFD.

***Effect of HFD, age, and sex on NE level in MA and plasma*.** NE concentration in MA was lower at 24-wk compared to 10- and 17-wk in male and female rats. In contrast, NE plasma levels were higher at 24-wk compared to 10- and 17-wk in male and female rats (Table 6). There was no significant effect of HFD in either sex.

Table 6. Quantification of NE from MA and plasma. HPLC measurement of tissue and plasma NE contents for male and female Dahl SS rats on CD and HFD for 10-, 17-, and 24-wk.

		Male			Female		
		10-wk	**17-wk**	**24-wk**	**10-wk**	**17-wk**	**24-wk**
Tissue NE (ng/g)	**CD**	**5127** ± 439 (n=9)	**5519** ± 462 (n=9)	**4259** ± 169 (n=15)	**7036** ± 707 (n=8)	**5788** ± 526 (n=7)	**3608** ± 368***** (n=12)
	HFD	**4998** ± 457 (n=13)	**5198** ± 261 (n=12)	**3603** ± 199 (n=11)	**5317** ± 243 (n=10)	**4897** ± 225 (n=6)	**3677** ± 348***** (n=13)
Plasma NE (pg/ml)	**CD**	**348** ± 19.6 (n=10)	**362** ± 23.9 (n=12)	**472** ± 20.4 (n=7)	**571** ± 35.0 (n=13)	**486** ± 36.6 (n=7)	**597** ± 68.8***** (n=8)
	HFD	**286** ± 9.9 (n=12)	**347** ± 16.0 (n=13)	**498** ± 35.9 (n=7)	**471** ± 20.9 (n=16)	**478** ± 50.5 (n=8)	**642** ± 47.1***** (n=7)

*****Indicates significant difference between 24- and 17-wk, and 24- and 10-wk of the same diet.

4.0 Discussion

Age, sex, and obesity are all known to affect blood pressure regulation and the development of hypertension (Sandberg and Ji, 2012; Buford, 2016; Leggio et al., 2017), but the mechanisms responsible are still being debated. Although one widely proposed mechanism is differences in sympathetic nervous system activity (da Silva et al., 2009; Straznicky et al., 2016), the objective of this study was specifically to investigate the possible differences in the effectiveness of sympathetic neurovascular coupling as an explanation. Relatively little attention has been given to possible changes in the effectiveness of neurovascular coupling in cardiovascular regulation because the process is difficult to evaluate in humans (Fink, 2018). We used a relatively new experimental model of hypertension which allowed us to analyze, separately and together, the impact of age, sex and obesity on both blood pressure and sympathetic neurovascular coupling in resistance arteries. Our main findings were: 1) BW and MAP were significantly higher in HFD- compared to CD-fed rats to a similar degree in both sexes; 2) neurogenic vasoconstriction of MA was transiently higher in HFD- versus CD-fed males during hypertension development, but not in females; however, it was greater at 17-wk compared to 10- and 24-wk in females regardless of diet; 3) adrenergic and purinergic components of vasoconstriction were not affected by HFD, age, and sex; 4) TH- and VNUT-ir nerve densities were not changed in HFD- compared to CD-fed rats in males and females; 5) NE content in MA tissue was not affected by HFD, but there was a slight decrease at 24-wk compared to 10- and 17-wk in both sexes; 6) Plasma NE level was higher at 24-wk compared to 10- and 17-wk in males and females and 7) vascular reactivity to exogenous NE and ATP was decreased with age in both

sexes and regardless of diet. In general, the data do not support the idea that alterations specifically in vascular sympathetic neurotransmission are responsible for obesity- or age- associated hypertension in our model in either males or females.

With regard to the effects of obesity, in one previous experimental study, HFD was associated with increased nerve-mediated vasoconstriction in MA from male Sprague Dawley rats (Haddock and Hill, 2011). Females were not investigated. Another study showed that EFS-induced adrenergic constriction was higher in obese male Wistar rats (Blanco-Rivero et al., 2011). Again, females were not studied. In our model, we showed amplified sympathetic neurotransmission to MA in HFD-fed male rats during the developmental phase of hypertension (17-wk), but not after hypertension was established. No such difference in HFD versus CD rats was observed in female rats. Although it is by no means certain that changes in neurovascular coupling affect hypertension development in HFD-fed male Dahl ss rats, it's worth considering possible mechanisms underlying the changes.

TH-ir nerve fiber density was quantified as an additional way of assessing adrenergic neurotransmission in MA from control and HFD fed rats. Previous studies using HFD-fed Sprague Dawley rat showed an increase in renal TH expression (Matthews et al., 2017), and sympathetic nerve density in MA (Haddock and Hill, 2011). A similar outcome was reported in studies of MA from young and old SHR (Kawamura et al., 1989). Moreover, increased sympathetic nerve density in the brain from stroke-prone SHR male rats is associated with severe HTN (Smeda, 1990). However, we did not find diet difference in TH nerve density in the present study. Lack of effect of HFD on the adrenergic (TH) nerve density is consistent with the absence of HFD effect on

NE content from MA. In contrast, NE content from MA has been shown to be lower in other hypertensive rat models (Luo et al., 2003). To our knowledge the TH-ir nerve density was not previously studied in female MA. However, others have shown increased TH expression in the hypothalamus in males and testosterone treated females compared to females fed a cafeteria diet (Plut et al., 2002). It's worth noting however that total NE content was not higher in HFD-fed versus CD-fed rats. Taken together, both TH-ir nerve density which marks adrenergic nerves and the NE content within the adrenergic nerves were not changed with HFD in males and females. These suggest that hypertension in the Dahl ss rat is not associated with changes in adrenergic neurotransmission.

Purinergic neuroeffector transmission plays an important role in maintaining vascular tone (Burnstock and Ralevic, 2014; Townsend et al., 2016). A previous study demonstrated that increased sympathetic transmission is mediated partly by enhanced purinergic neurotransmission in obese male Sprague Dawley rats (Haddock and Hill, 2011). Furthermore, they showed that both adrenergic and purinergic components were augmented in obese rats at 3 and 5 Hz EFS. Interestingly, at higher frequency (10 Hz) only the purinergic proportion was increased in obese rats indicating that purinergic transmission dominates at high frequency in obese male rats. However, in our study the purinergic component in the obese males was stimulated at higher EFS (20 Hz) than what was used in the previous study (10 Hz). Despite the high frequency stimulation, our finding shows that the adrenergic component was overall dominant regardless of the diet. Similar results occur in female rats.

A study of the tail artery from diabetic rats showed decreased vascular reactivity to NE and ATP (Speirs et al., 2006), which is similar to our data although our rats are not diabetic. However, these data disagree with the increase in adrenergic reactivity previously reported in obese male Zucker rats (Stepp and Frisbee, 2002). Similarly, there was no difference in vasoconstriction (obese vs. control) to exogenous NE in MA from male Wistar rats (Blanco-Rivero et al., 2011). In regards to ATP, there are studies from the renal vasculature showing increased reactivity in male Wistar and SHR hypertensive rats that is inconsistent with our data (Harris, 1972; Fernandez et al., 2000). However, there are no similar studies in female rats. The discrepancy in the vascular response to direct nerve stimulation and to exogenous NE and ATP could be due to the following reasons. First, when periarterial nerve is stimulated it releases neuropeptide Y (NPY) along NE and ATP, and NPY is another vasoconstrictor known to be involved in hypertension (Westfall, 2006). Thus, NPY enhances the vascular response to the nerve-mediated as compared to individually applied NE and ATP. Second, exogenous NE and ATP are dispersed throughout the tissue including to extra junctional receptors (Hotta, 1969; Itoh et al., 1983). Therefore, they will not have as quick and intense response as the neurotransmitters released within a very short distance from the postjunctional receptors.

With regard to sex and aging effects on neurovascular transduction, a recent thorough study in humans demonstrated that neurovascular transduction increases with age in women but decreases in men (Briant et al., 2016). Another study showed no relationship between resting sympathetic activity and MAP or vascular conductance in young females in comparison to males (Robinson et al., 2019). In general, human

studies support a gradual increase in the effectiveness of neurovascular coupling with age in women and a decrease in men; we did not observe this pattern in our model. This could represent a species-related effect. For example, EFS-induced adrenergic constriction was shown to decline with age in Fisher 344 female rats (Sullivan and Davison, 2001). Alternatively, methodological differences could be a factor, since all experimental studies were in isolated vessels *in vitro*, whereas human experiments were *in vivo*.

Our group published a report showing that ganglionic blockade of the sympathetic nerves contributes to a greater depressor effect in MAP in HFD-fed males than HFD-fed females (Fernandes et al., 2018). In the present study, we also determined sympathetic activity indirectly by measuring plasma NE concentration. That measure showed no differences between HFD and CD rats, with similar small increments during aging in male and female animals. This suggests that HFD-induced hypertension may be caused by multiple mechanisms in which the sympathetic nervous system is only one of them.

Development of HFD-induced hypertension in Dahl ss rats is similar in males and females, being of similar magnitude, gradual in onset and accompanied by relatively modest increments in body weight in both sexes. In females, but not males, diet-independent hypertension may be driven in part by a transient increase in sympathetic neurotransmission originating from regions other than the neuroeffector junction in MA. Therefore, the mechanism by which transient increase in the neurogenic constriction in females despite no change in NE content and release as well as in vascular reactivity to adrenergic transmitters cannot be identified with certainty.

CHAPTER 5: EFFECTS OF HIGH FAT DIET ON SYMPATHETIC NEUROVASCULAR TRANSDUCTION IN MESENTERIC VEIN FROM DAHL SALT-SENSITIVE RAT

1.0 Abstract

Mesenteric veins (MV) hold large amounts of blood. Increased sympathetic neurotransmission increases venous return, and cardiac output. More blood is redistributed from compliant veins to resistance arteries causing elevation in total peripheral resistance and blood pressure. The sympathetic neurovascular transduction in relation to obesity in MV is not adequately studied. We tested the hypothesis that sympathetic neurotransmission is higher in HFD compared to CD from MV in males but not females in Dahl ss rat. MV constriction was measured by pressure myography, and electrical field stimulation (EFS, 0.2 – 20 Hz). Emax was greater in HFD compared to CD in 17-wk males. There was no difference in adrenergic and purinergic components between diets, ages or sexes. Venous reactivity to exogenous NE (10^{-9} – 10^{-5} M) was greater in HFD vs. CD at 17- and 24-wk in males whereas it was lesser at similar times in females. Venous reactivity to exogenous ATP (10^{-8} – $3x10^{-3}$ M) was greater in HFD versus CD at 10-wk but lesser at 17-wk in males. Venous reactivity to exogenous ATP was lower in HFD versus CD at 10-, 17-, and 24-wk in females. TH and VNUT nerve count (density) were not changes in response to HFD in males and females. MV NE content decreased with age while plasma level increased with age. Taken together, these data suggest that HFD-induced hypertension is not driven by increase in sympathetic neurotransmission in the MV in males and females.

2.0 Introduction

Obesity is a major health risk indicator that is associated with several metabolic disorders such as hypertension (Hall et al., 2015). Obesity-related hypertension is a leading cause for cardiovascular disease, stroke, kidney failure, and diabetes; it is mainly caused by sympathetic overdrive (Landsberg et al., 2013). Several studies from experimental animals (Judy et al., 1976; Park et al., 2010; Haddock and Hill, 2011; Kandlikar and Fink, 2011), and humans (Grassi, 1998; Joyner et al., 2010; Baker et al., 2016) have established the link between increase in peripheral sympathetic neurotransmission and elevation in blood pressure. Thus, the circulation of the splanchnic organs is important not only in hemodynamics, but also in blood pressure control (Greenway, 1983; Fudim et al., 2017).

MVs are part of the splanchnic circulation which contain close to 60% of circulating blood (Fink, 2009). This is mainly due to the anatomical structure of MVs. MVs have thinner layer of vascular smooth muscle cells (VSMCs), and wider lumen diameter than mesenteric arteries, for example, and these make them suitable as blood reservoirs. This characteristic is known as venous capacitance (Tyberg, 2002; Gelman, 2008). Venous capacitance is the ability for veins to store blood. Mean circulatory filling pressure (MCFP) measures the distending pressure in veins. This is measured by momentarily halting the heart from pumping blood (Rothe, 1986). Decrease in venous diameter (decrease in capacitance) leads to increase in venous return to the heart that eventually increases cardiac output. Moreover, the redistribution of blood from the MVs to the peripheral arteries increases total peripheral resistance. The increases in the cardiac output and total peripheral resistance then result in increase in arterial blood

pressure (Martin et al., 1998; Mayet and Hughes, 2003). Therefore, venoconstriction and venodilation are important mechanisms by which the homeostasis of cardiac output and blood pressure are maintained (Rothe, 1986).

Perivenous sympathetic nerves co-release NE and ATP into the neuroeffector junction that act on adrenergic and purinergic receptors, respectively on the VSMCs (Bobalova and Mutafova-Yambolieva, 2001). NE binds to α1-AR inducing vasoconstriction; however, there are conflicting reports on the types of purinergic receptors in the VSMCs from MVs. Some report that there is P2X receptor localized in MVs from adult rats (Hansen et al., 1999), but other studies indicate that only the P2Y receptor is found in MVs from normotensive and hypertensive rats (Galligan et al., 2001; Burnstock, 2017). In either case, venous tone is maintained by sympathetic nerves, and increased of sympathetic neurotransmission decreased venous capacitance leading to hypertension (Pang, 2001). The relationship between changes in venous tone and hypertension is well established in studies from experimental models of hypertension (Simon, 1976; Martin et al., 1998). These studies have demonstrated that a decrease in venous capacitance (increase in venous resistance) is directly associated with increased blood pressure.

The contribution of veins in general and MVs in particular to blood pressure regulation is fairly understood; however, more work is needed to explore and understand the effect of obesity in male and female rats. Specifically, understanding the effect of high fat diet on the sympathetic neurotransmission, and how the venous tone responds to such treatment in males and females will further our understanding of obesity-related hypertension. Previous studies in animals and humans have shown sex

differences in obesity-associated hypertension and sympathetic nerve activity (Brooks et al., 2015; Faulkner and Belin de Chantemele, 2018; Fernandes et al., 2018), but the underlying mechanisms for such disparity is not well understood. This led us to hypothesize that sympathetic neurotransmission increases more in male than female Dahl ss rats that are on HFD.

3.0 Results

***Effects of diet and age on nerve-mediated venous constriction*.** Because venous tone plays important role in regulating blood pressure, we tested the effect of HFD on venous response to a direct perivenous nerve stimulation (Fig. 25a and b). The maximum response (Emax) from EFS (0.2 - 20 Hz) was increased in HFD male rats at 17-wk, but the overall trend shows an age-related decrease in Emax but was slightly increased with HFD in males (Fig. 25c). There was no change in Emax in females (Fig. 25d). The frequency for the half-maximal response (S_{50}) slightly decreases with age and irrespective of diet in both sexes (Fig. 25e and f). These show a curve-shift to the left, which means increased venoconstriction with age.

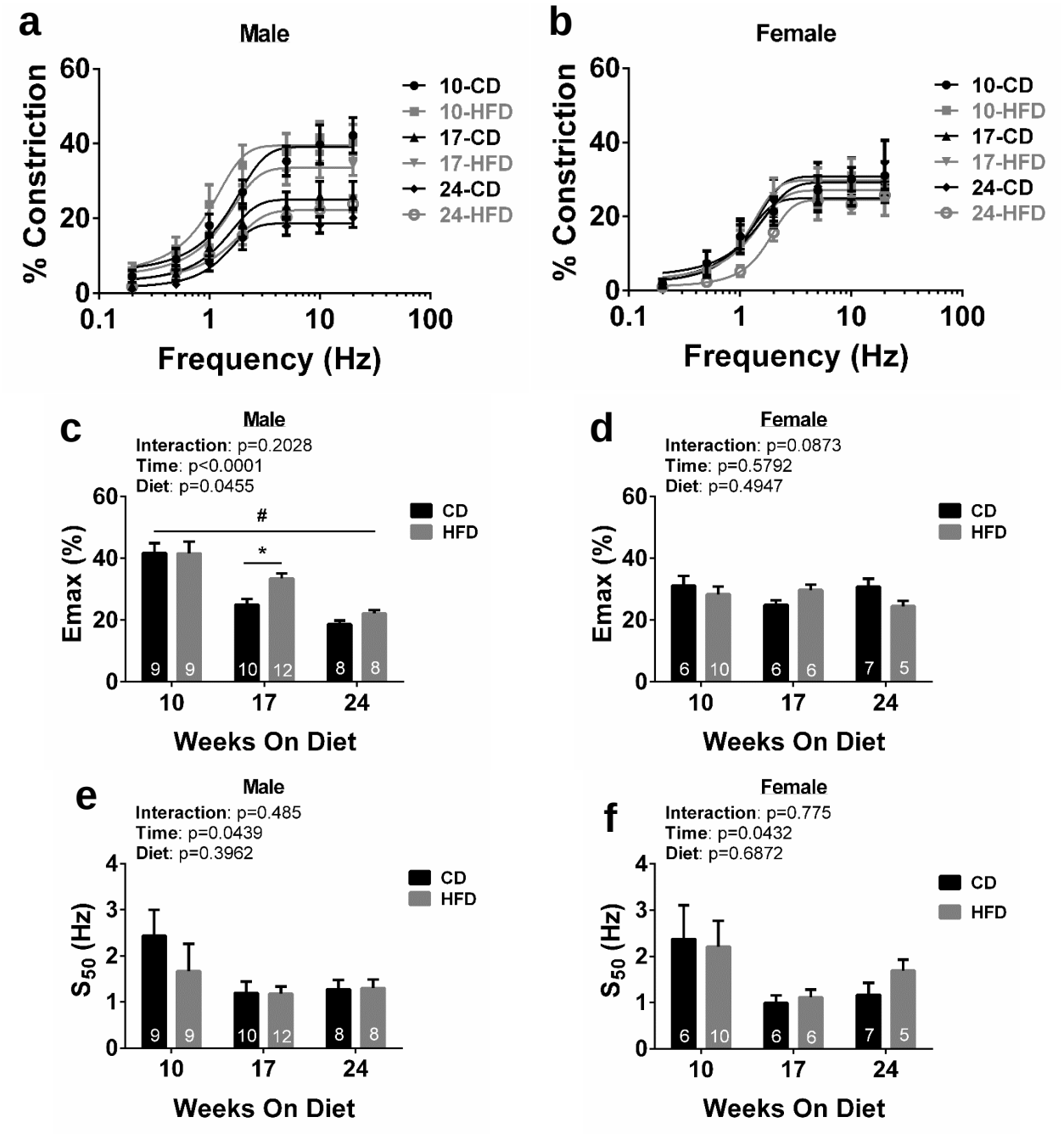


Figure 25. **Vasoconstriction in response to direct perivenous stimulation**. ***a***: Frequency response curves for male rats. ***b***: Frequency response curves for female rats. ***c***: Maximum frequency response (Emax) compared between CD and HFD at 10-, 17-, and 24-wk in males. *****Emax is higher in HFD versus CD at 17-wk. #Emax is lower at 24-wk than at earlier times (in both diets). ***d***: Emax compared between CD and HFD at 10-, 17-, and 24-wk in females. ***e***: Frequencies for the half maximum constriction (S_{50}) compared between CD and HFD at 10-, 17-, and 24-wk in males. ***f***: S_{50} compared between CD and HFD at 10-, 17-, and 24-wk in females.

***Effect of UK 14304 on neurogenic transmission in veins*.** UK 14304 is an agonist for α2-AR located on the nerve terminal that inhibits neurotransmitters (NE and ATP) release leading to decrease in vasoconstriction. Thus, using 10 Hz EFS and UK 14304 (10^{-9} - $3x10^{-6}$ M) we examined whether the function of α2-AR is affected by HFD (Fig. 26a and b). The maximum inhibition (Imax) does not change in males (Fig. 26c); however, it increases in 17-wk HFD females (Fig. 26d). In addition, there is an overall increase in Imax in the HFD females. The concentration of UK 14304 that produces half-maximum of inhibition (IC_{50}) does not change in males (Fig. 26e); yet, it increases in 17-wk HFD females (Fig. 26f).

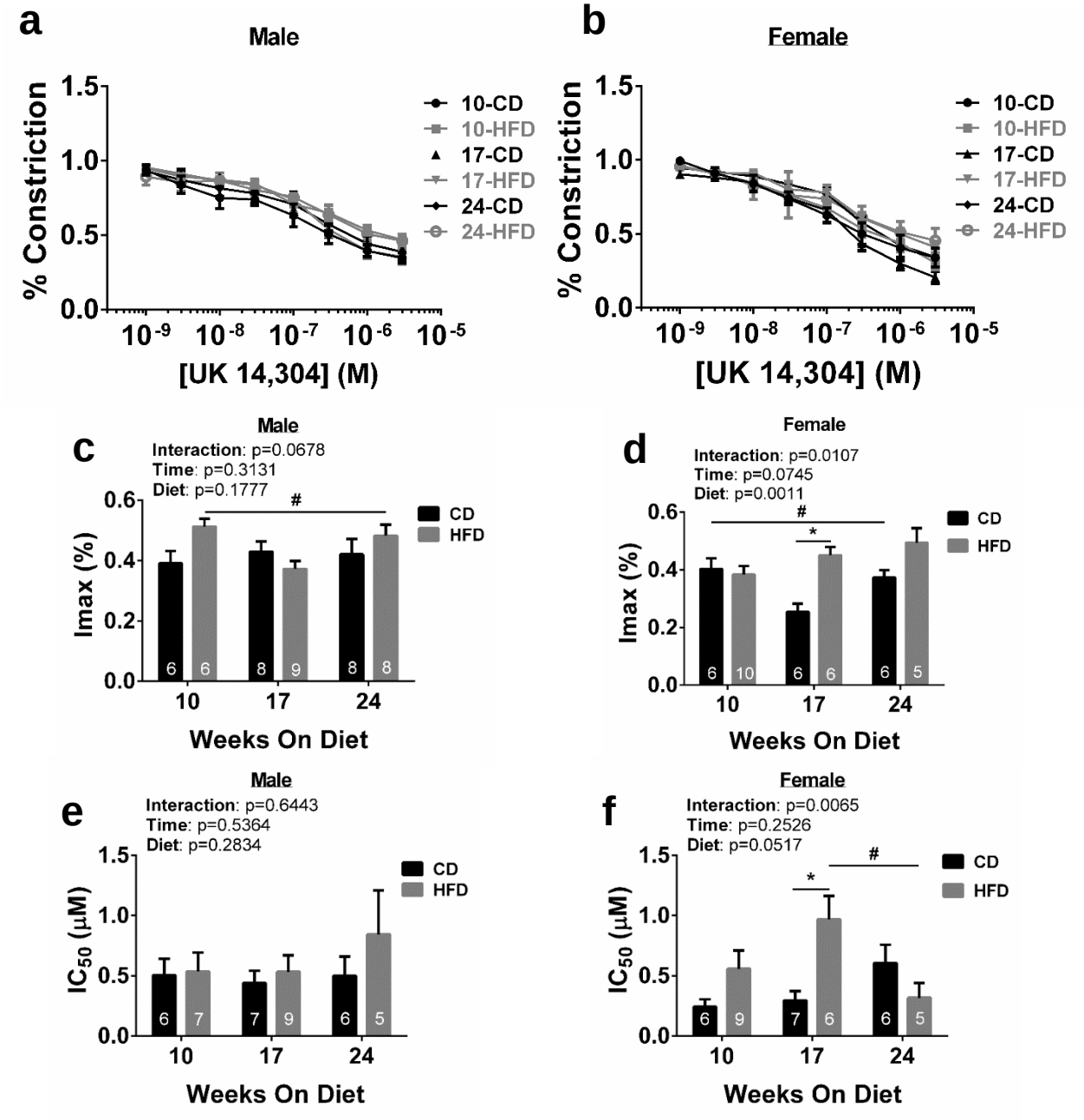


Figure 26. Inhibition of venous constriction by activating α2-AR. ***a***: % constriction response to increasing concentration of UK 14,304 in male rats. ***b***: % constriction response to increasing concentration of UK 14,304 in female rats. ***c***: Imax for % constriction in response to increasing UK 14,304 in males. #Imax is lower in HFD at 17-wk compared to HFD at10- and 24-wk. ***d***: Imax for % constriction in response to increasing UK 14,304 in females. *****Imax is greater in HFD versus CD at 17-wk. #Imax is lower in CD at 17-wk compared to CD at the other times. ***e***: IC_{50} for % constriction in response to increasing UK 14,304 in males. ***f***: IC_{50} for % constriction in response to increasing UK 14,304 in females. *IC50 is greater in HFD compared to CD at 17-wk. #IC50 is lower in HFD at 24-wk compared to HFD at 17-wk.

***Effect of Cocaine on neurogenic venoconstriction*.** Cocaine is an antagonist for the NET that leads to increase in vasoconstriction. Therefore, we tested if NET is affected by cocaine with EFS (0.2 - 20 Hz). Emax was higher in cocaine perfused HFD rats compared to cocaine free Krebs perfusion HFD at 10-, 17-, and 24-wk in males. There was also greater Emax in Cocaine perfused response in CD versus CD without cocaine perfusion (Fig. 27a). In females, Emax was higher in cocaine perfused responses compared to unperfused tissues at all time-points except in CD at 24-wk (Fig. 27b). There was no effect from cocaine on S_{50} in male and female rats (Fig. 27c, d).

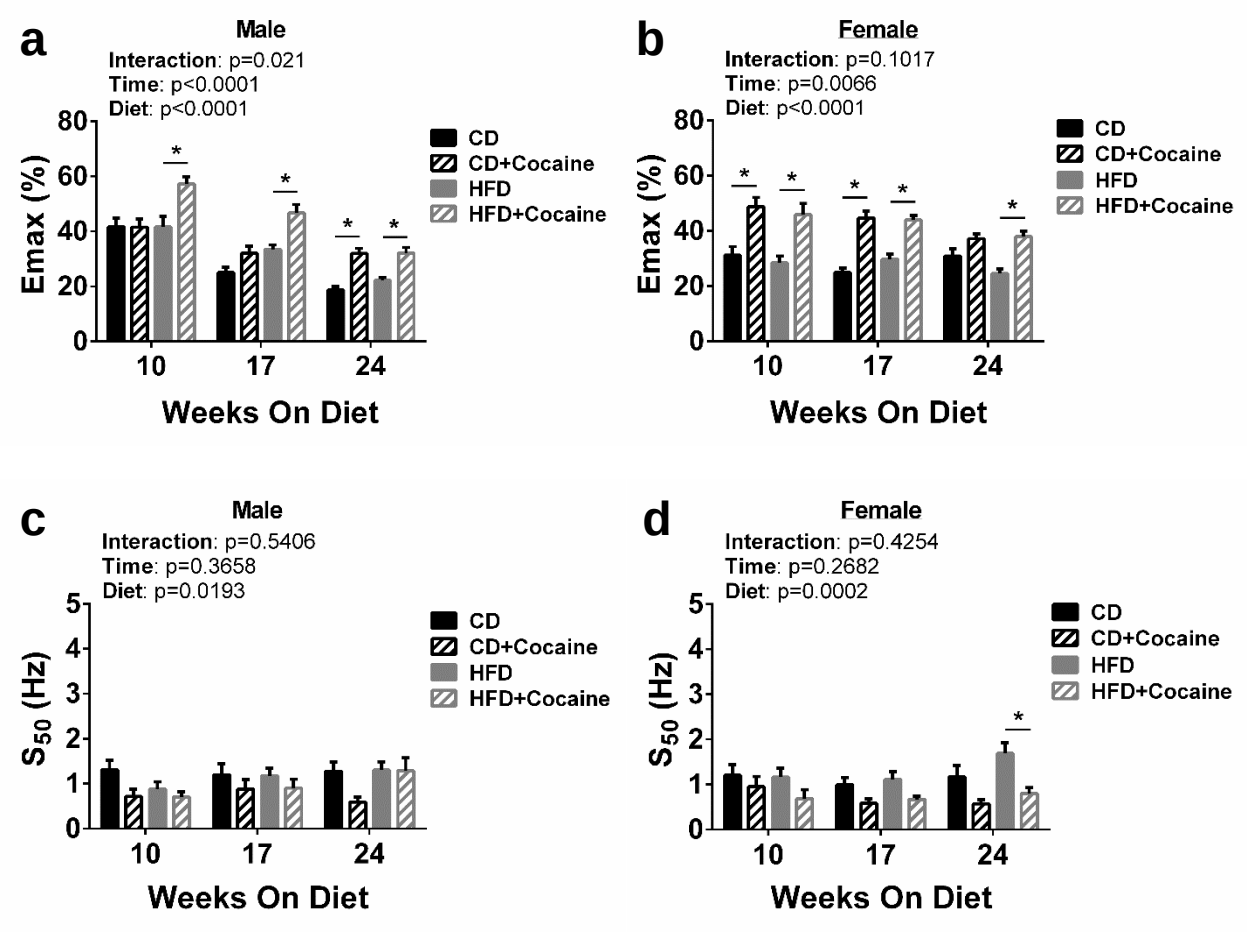


Figure 27. Cocaine-induced change in venous constriction. ***a***: Emax evaluated in cocaine perfused and cocaine free Krebs solution in males. ***b***: Emax evaluated in cocaine perfused and cocaine free Krebs solution in females. ***c***: S_{50} for cocaine perfused and cocaine free Krebs solution in males. ***d***: S_{50} cocaine perfused and cocaine free Krebs solution in females. *Indicates significant change between groups in response to cocaine.

***Composition of adrenergic and purinergic components in mesenteric vein*.** Neurogenic transmission constitutes adrenergic and purinergic components. Adrenergic transmission is mediated by NE binding to α1-AR in the VSMCs. We used α1-AR antagonist (Prazosin, 0.1 μM; Fig. 28b) with 10 Hz EFS (Fig. 28a) to test the contribution of the adrenergic component. Our finding shows that HFD does not affect the adrenergic component (~60%) at all time-point in male and female rats (Fig. 28e, f). Purinergic transmission, on the other hand, is mediated by ATP that binds to P2Y receptor on the VSMCs. We used an antagonist for P2Y (Suramin, 100 μM; Fig. 28c) in addition to Prazosin and stimulated at 10 Hz EFS to test the contribution of the purinergic component. Our finding indicates that HFD does not affect the purinergic component (~20%) at any time-point in males (Fig. 28g). Tetrodotoxin (TTX, 0.3 μM) was used to verify that the EFS-induced venoconstriction was nerve-mediated (~90%) (Fig. 28d). The remaining 10% is non-neurogenic constriction due to direct stimulation of the VSMCs, and it was completely blocked by Nifedipine (antagonist for L-type Ca^{2+} channels, 1 μM) (not shown).

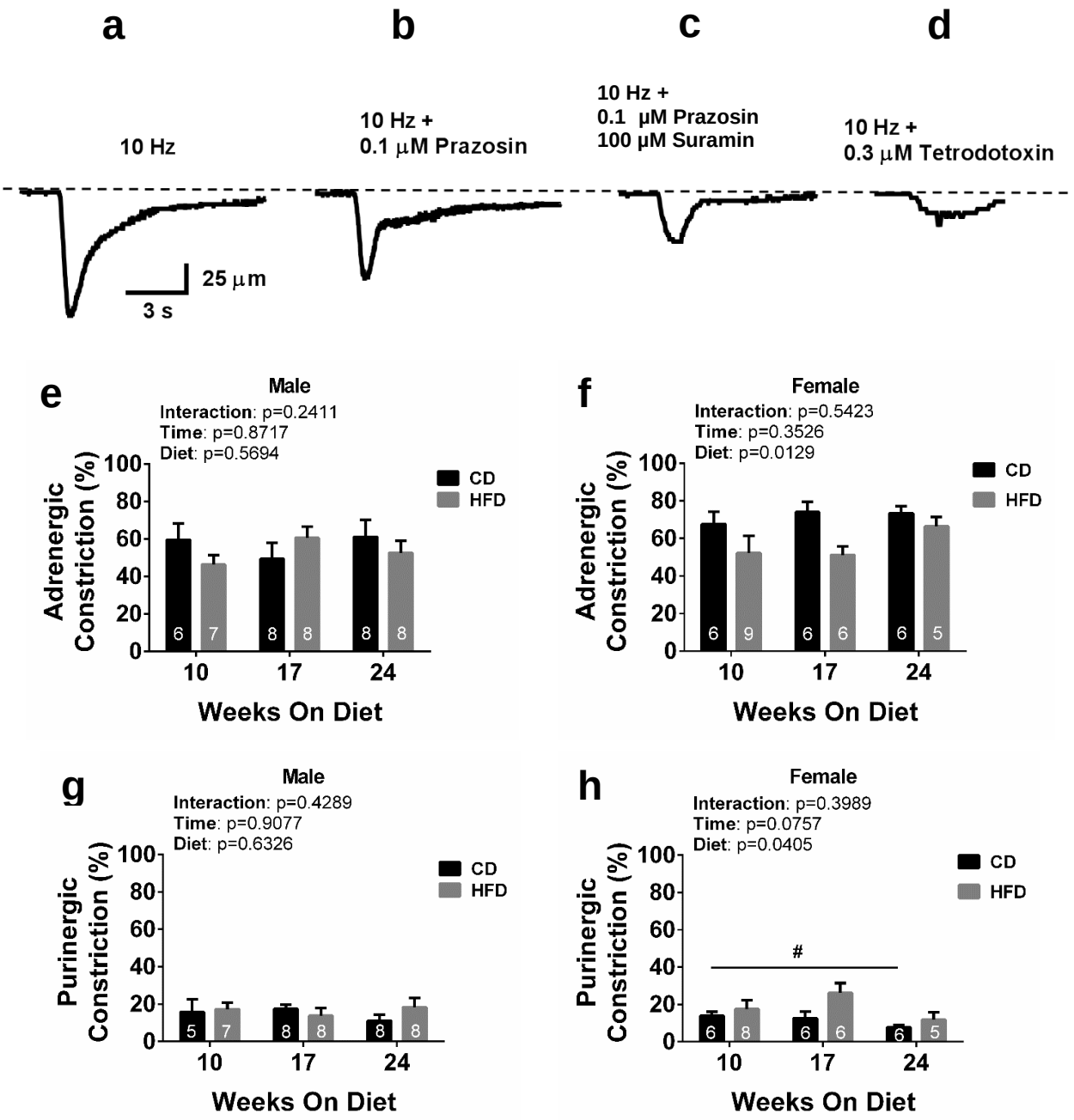


Figure 28. **Proportion of adrenergic and purinergic constriction in MV**. Representative traces of nerve stimulation. ***a***: 10 Hz. ***b***: 10 Hz + 0.1 μM Prazosin. ***c***: 10 Hz + 0.1 μM Prazosin + 100 μM Suramin. ***d***: 10 Hz + 0.3 μM Tetrodotoxin. ***e***: Adrenergic proportion compared between CD and HFD at 10-, 17-, and 24-wk in males. ***f***: Adrenergic proportion compared between CD and HFD at 10-, 17-, and 24-wk in males. ***g***: Purinergic proportion compared between CD and HFD at 10-, 17-, and 24-wk in males. ***h***: Purinergic proportion compared between CD and HFD at 10-, 17-, and 24-wk in females. #Purinergic component is lower in CD at 24-wk compared to CD at 10-wk.

***Venous reactivity to exogenous NE*.** Like endogenous NE, exogenous NE induces vasoconstriction. We evaluated venoconstriction to increasing NE (10^{-9} – 10^{-5} M; Fig. 29a and b). Emax was decreased in HFD in male rats; Emax was also decreased with age in both sexes (Fig. 29c and d). Interestingly, EC_{50} in HFD rats was decreased in males at 17-, and 24-wk, and overall but was increased at 17-and 24-wk (Fig. 29e). In contrast, EC_{50} in females was increased at 17-and 24-wk, and overall. EC_{50} was also increased with age in female rats (Fig. 29f). Moreover, there was diet-time interaction that influenced EC_{50} in male (Fig. 29e) and female rats.

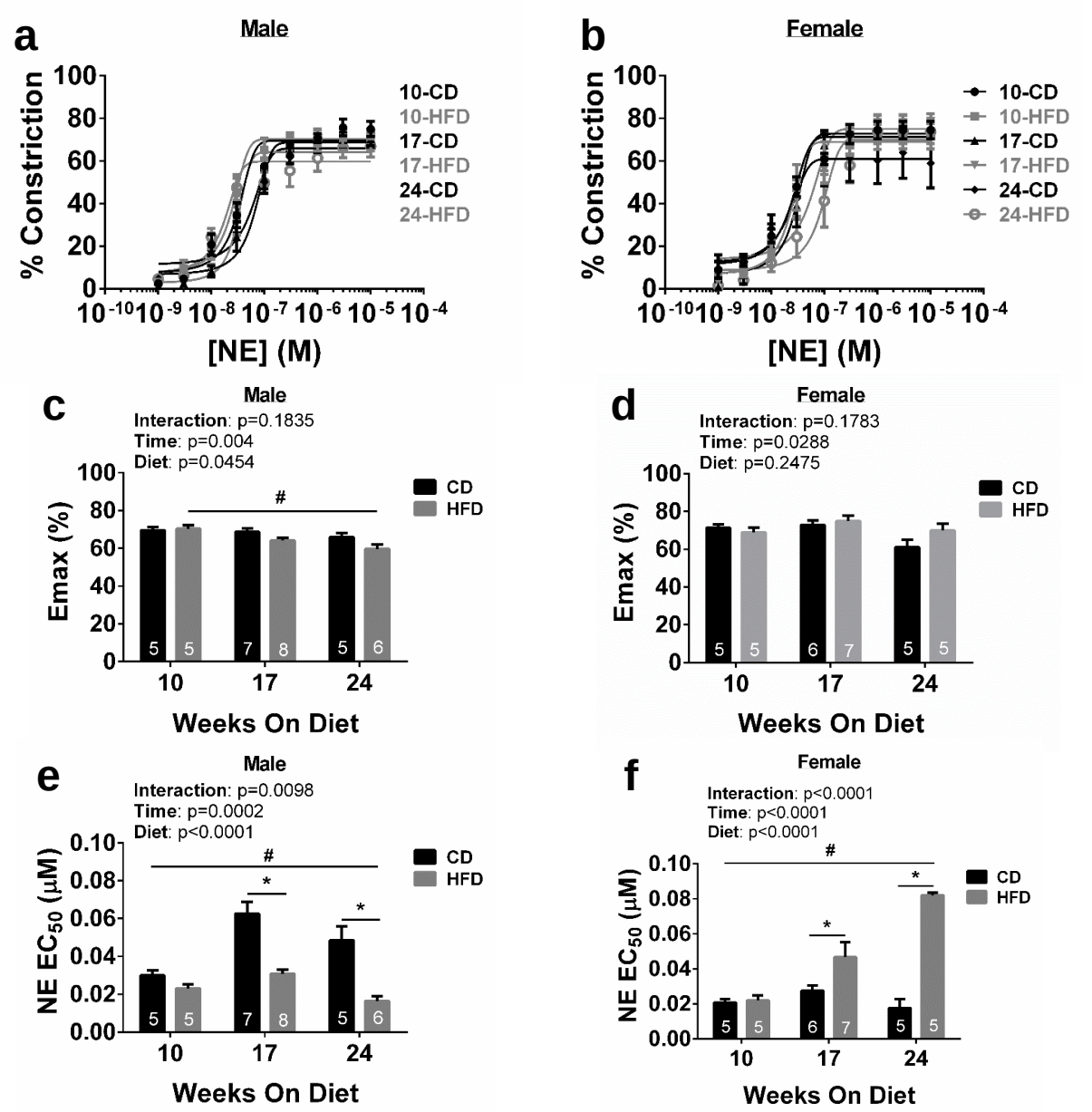


Figure 29. **Venous reactivity to exogenous NE**. ***a***: NE concentration-response curves for males. ***b***: NE concentration-response curves for females. ***c***: Emax for NE concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in males. #Emax is lower in HFD at 24-wk compared to HFD at 10-wk. ***d***: Emax for NE concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in females. ***e***: EC_{50} for NE concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in males. *****EC50 is lower in HFD compared to CD at 17- and 24-wk. #EC_{50} is lower in CD at 10-wk versus CD at later times. ***f***: EC_{50} for NE concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in females. *****EC_{50} is higher in HFD versus CD at 17- and 24-wk. #EC_{50} is greater in HFD at 24-wk compared to HFD at earlier times.

***Venous reactivity to exogenous ATP*.** Like endogenous ATP, exogenous ATP induces vasoconstriction. We evaluated venoconstriction to increasing ATP (10^{-8} – $3x10^{-3}$ M; Fig. 30a and b). Consequently, Emax was decreased at 17-wk in males (Fig. 30c) whereas it was increased in HFD-fed females (Fig. 30d). EC_{50} in HFD rats was decreased in males at 10-wk but was increased at 17-wk and decreased overall. EC50 was also decreased at 17-wk but was increased at 24-wk compared to 10-wk in males (Fig. 30e). In females EC_{50} was increased at all time-points in HFD compared to CD rats (Fig. 30f).

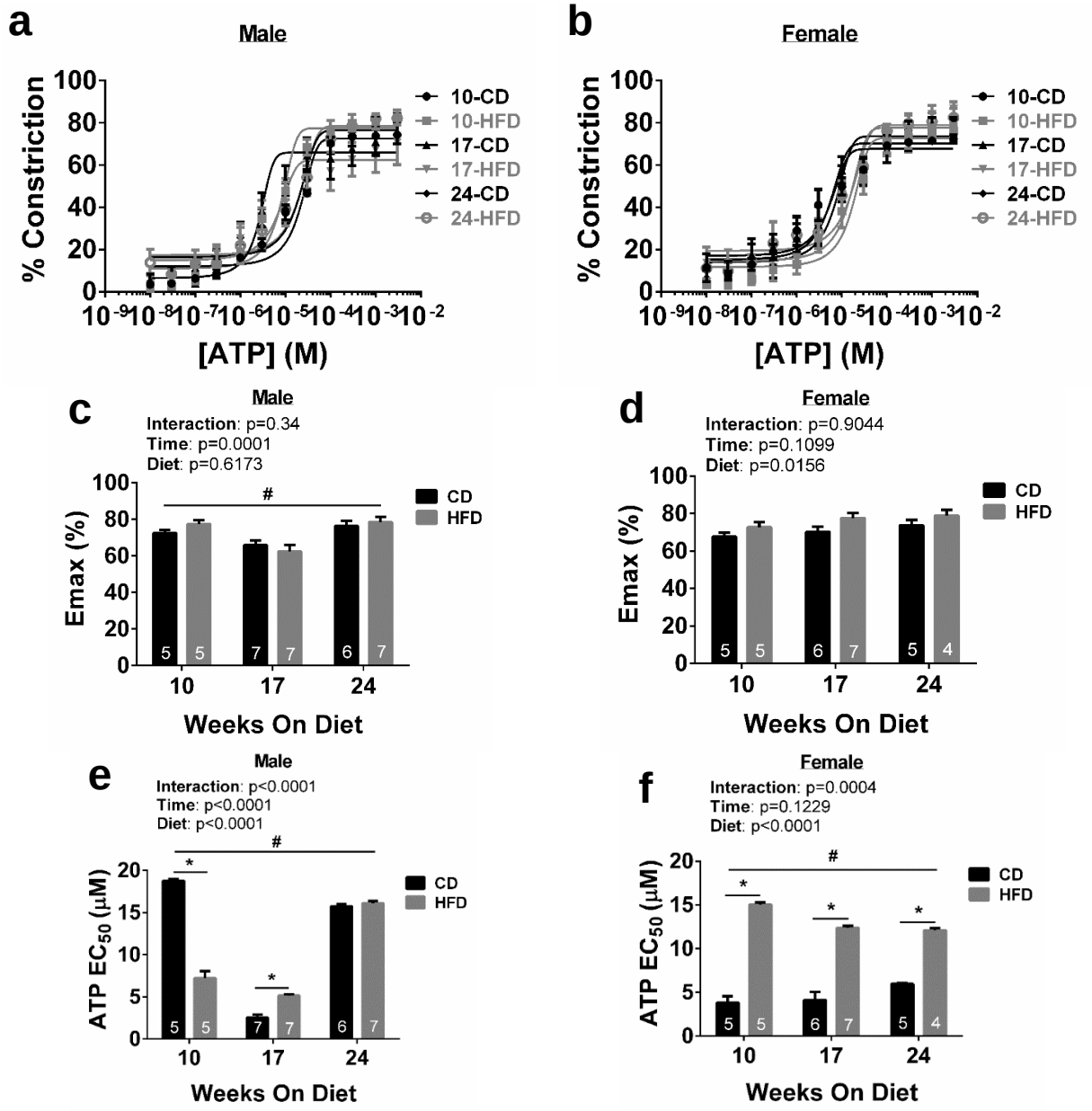


Figure 30. **Venous reactivity to exogenous ATP**. ***a***: ATP concentration-response curves for males. ***b***: ATP concentration-response curves for females. ***c***: Emax for ATP concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in males. #Emax is lower in HFD at 17-wk compared to HFD at 10-and 24-wk. ***d***: Emax for ATP concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in females. ***e***: EC_{50} for ATP concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in males. *****EC50 is lower in HFD versus CD at 10-wk, but greater in HFD vs CD at 17-wk. #EC_{50} is lower at 17-wk compared to the other times in both diets. ***f***: EC_{50} for ATP concentration-response curve compared between CD and HFD at 10-, 17-, and 24-wk in females. *****EC_{50} is higher in HFD versus CD at 10-, 17-, and 24-wk. #EC_{50} is higher at 10-wk in HFD compared to HFD at the other times.

***Effect of HFD on TH-ir nerve fiber density*.** We localized TH-ir nerve fibers surrounding the veins using immunofluorescent procedure followed by confocal imaging. This allowed us to acquire z-series sections of images with high resolution. Representative micrographs are shown for males and females in Fig. 31a and b, respectively. Then, we used ImageJ for density analysis. Our findings demonstrate that TH nerve count was not affected by HFD in males (Fig. 31c) and females (Fig. 31d).

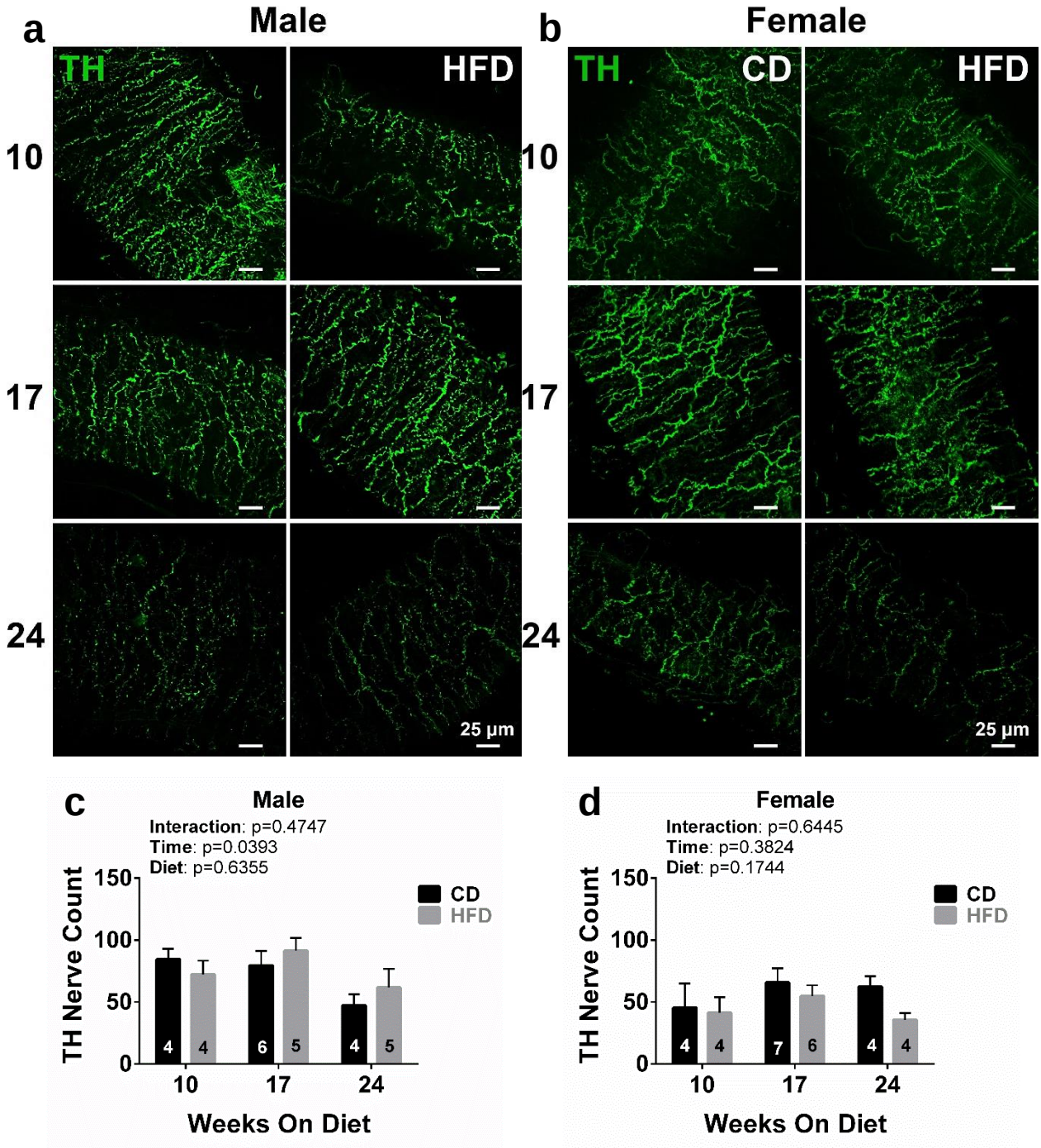


Figure 31. **Perivenous TH nerve density.** ***a***: Representative TH-ir nerve fiber immunofluorescent confocal images in male rats. ***b***: Representative TH-ir nerve fiber immunofluorescent confocal images in female rats. ***c***: TH-ir nerve fiber density shown in CD and HFD at 10-, 17-, and 24-wk in males. ***d***: TH-ir nerve fiber density shown in CD and HFD at 10-, 17-, and 24-wk in females.

***Effect of HFD on VNUT-ir nerve fiber density*.** Like TH, we localized VNUT-ir nerve fibers surrounding the veins using immunofluorescent procedure followed by confocal imaging. This allowed us to acquire z-series sections of images with high resolution. Representative micrographs are shown for males and females in Fig. 32a and b, respectively. Then, we used ImageJ for density analysis. Our findings reveal that HFD did not change VNUT nerve fiber count in males (Fig. 32c) and females (Fig. 32d).

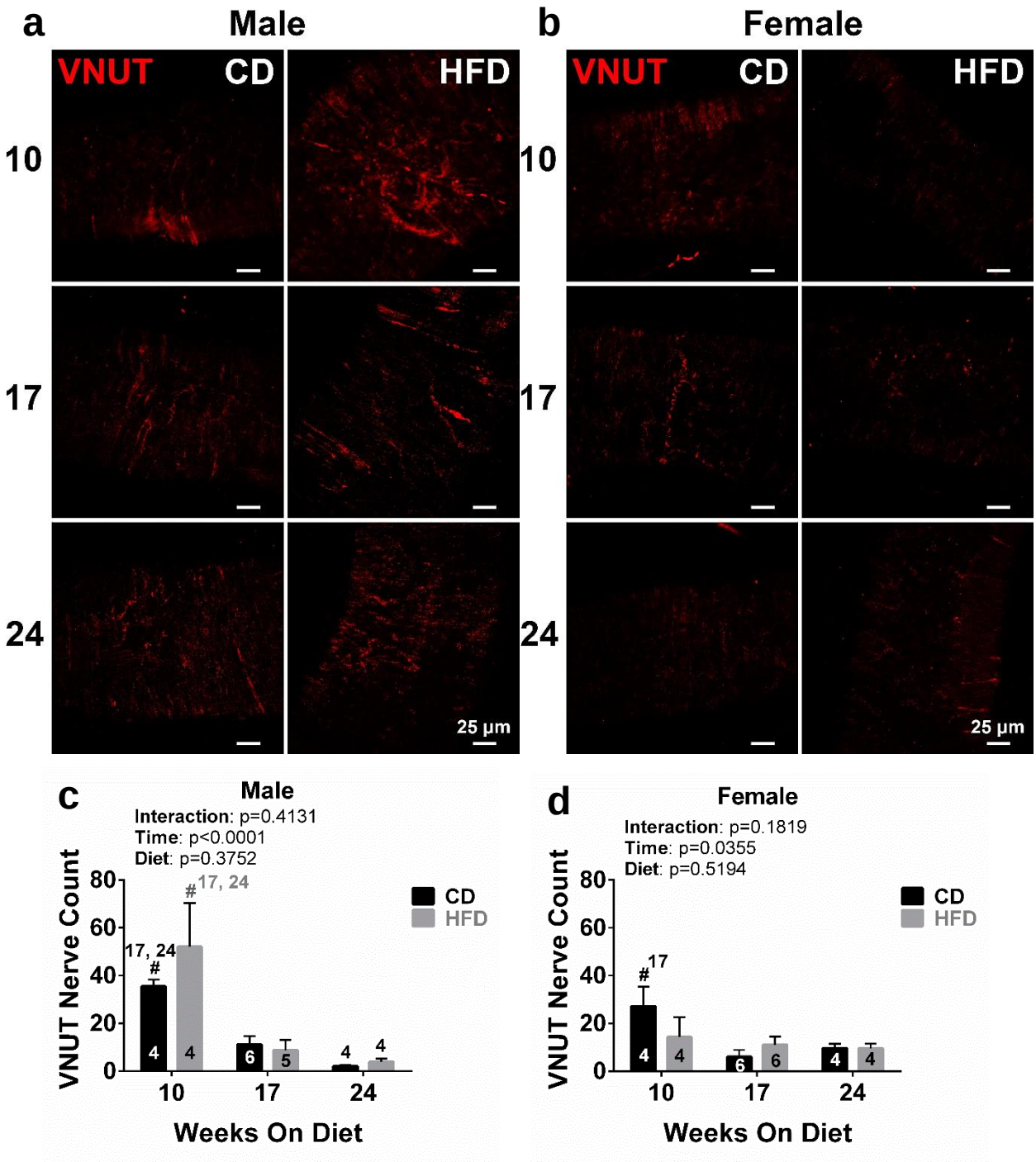


Figure 32. **Perivenous VNUT nerve density.** ***a***: Representative VNUT-ir nerve fiber immunofluorescent confocal images in male rats. ***b***: Representative VNUT-ir nerve fiber immunofluorescent confocal images in female rats. ***c***: VNUT-ir nerve fiber density shown in CD and HFD at 10-, 17-, and 24-wk in males. #VNUT-ir nerve count is greater at 10-wk compared to later times in both diets. ***d***: VNUT-ir nerve fiber density shown in CD and HFD at 10-, 17-, and 24-wk in females. #VNUT-ir nerve density is greater in CD at 10-wk compared to 17-wk in CD in females.

***Effect of HFD on venous and plasma NE contents*.** Measurement of NE level in veins and plasma are indicators of changes in sympathetic neurotransmission. Hence, we measured NE contents of MV tissue and plasma by HPLC. Our findings show that there was no difference in NE content between CD and HFD rats in males. There was, however, a decrease at 24-wk (Table 7). In females, tissue NE level was decreased in HFD rats at 10-wk. There was also an overall decreasing trend associated with age (Table 7).

Table 7. **Quantification of NE from MV and plasma**. Shows MV and plasma NE level measured by HPLC.

		Male			**Female**		
		10-wk	**17-wk**	**24-wk**	**10-wk**	**17-wk**	**24-wk**
Tissue NE (ng/g)	**CD**	**5127** ± 439 (n=9)	**5519** ± 462 (n=9)	**4259** ± 169 (n=15)	**7036** ± 707 (n=7)	**5788** ± 526 (n=8)	**3608** ± 368 (n=10)
	HFD	**4998** ± 457 (n=12)	**5198** ± 261 (n=12)	**3603** ± 199 (n=11)	**5317** ± 243 (n=10)	**4897** ± 225 (n=8)	**3677** ± 348 (n=11)

Data are shown as means ± SEM, and number of animals used are: CD= 7-15; HFD=8-12 for tissue NE, and CD= 7-13; HFD=7-16 for plasma NE.

4.0 Discussion

The present study investigated the effect of HFD on the sympathetic neurotransmission and potential mechanisms responsible for HFD-induced hypertension in MV. Our main findings include: 1) Neurogenic constriction is slightly greater in HFD versus CD at 17-wk in males; 2) α2-AR is not impaired by HFD; 3) NET is not impaired by HFD; 4) Adrenergic and purinergic constrictions are not affected by HFD; 5) Emax for NE response curve decreases with age in both sexes and decreases with HFD in males only. In contrast, EC_{50} increases with age in both sexes and decreases with HFD in males but increases with HFD in females; 6) Venous reactivity to NE increased with HFD compared to CD in males while it decreased with HFD compared to CD in females; 7) Compared to CD, HFD did not change TH and VNUT nerve densities in males and females; 8) NE content in MV tissue decreases at 24-wk compared to earlier time points while plasma NE level increases at 24-wk compared to 10- and 17-wk in males and females.

Consumption of HFD over time has been known to lead to obesity and obesity-associated hypertension (Nagae et al., 2009; Hariri and Thibault, 2010; Marques et al., 2016; Taylor et al., 2018). In this study HFD induced body weight gain, but not necessarily obesity because there is only 10-15% difference in body weight between the HFD-fed and CD-fed rats. We were also interested to see whether HFD would have different effect on body weight and hypertension in males and females. Our findings suggest that the cause of hypertension is not linked to HFD caused increase in sympathetic neurotransmission in the MV neuroeffector junction. We did not observe

sex differences in our data although others have reported that males are more prone to HFD-associated hypertension than females (Tamaya-Mori et al., 2002).

The reduction in the neurogenic Emax with respect to age in males suggests that there may be age-related changes in the postjunctional receptors (Ren et al., 1996). These changes could involve downregulation of receptor expression (Passmore et al., 2005) or other structural changes (Seawright et al., 2018). In contrast, the increase in Emax in the HFD males shows that the vasodilatory mechanism mediated by nitric oxide (Higashi et al., 2001) and/or β receptor (Schutzer et al., 2006; Schutzer and Mader, 2012) could have been rendered less functional. Moreover, there may have been receptor upregulation, for example- α1-AR, in favor of increasing vasoconstriction outcome. It is also possible that the sensitivity of α1-AR is increased in the kidney since the kidney in this rat is somehow damaged (mainly in males) (Sprick et al., 2019), and ageing might exacerbate it. Some studies, however, report that advancing age does not affect vascular response (Duckles et al., 1985; Duckles, 1987). Taken together, HFD did not have any significant and consistent effect on the neurogenic response in males and females.

Cocaine was used to test the effect of the NET in MV. Our group have previously shown that Cocaine treatment increases vasoconstriction in DOCA-salt rats (Park et al., 2006). Our present findings in the Dahl ss rats are consistent with that study, and it suggests that NET is not impaired with age or HFD in MV. Furthermore, this shows that the HFD-induced hypertension shown in this study is not related to changes in NET in MV.

Previous studies from DOCA-salt hypertensive rats have shown that prejunctional α2-AR impairment by macrophages leads to hypertension in MA (Thang et al., 2015; Mui et al., 2018). We tested whether HFD and age would produce similar effects in Dahl ss rats. Our data show an increase in the maximum inhibition to venous constriction suggesting that prejunctional α2-AR is not impaired in this animal model. We also tested dietary effect to the contributions of adrenergic and purinergic venoconstriction. Our data show that adrenergic and purinergic constrictions did not change in response to HFD in males and females. On the other hand, our data indicates that adrenergic constriction is plays dominant role than purinergic constriction by about three-fold. This is consistent with previous studies that have shown that adrenergic neurotransmission plays dominant role in venoconstriction (Hiraoka et al., 2000; Carter and Ludwig, 2008; Park et al., 2010).

Sympathetic neurotransmission increases with obesity, and such increase is attributed to hypertension (Faulkner and Belin de Chantemele, 2018). NE is one of the main neurotransmitters that causes venoconstriction in the mesenteric circulation (Xu et al., 2007). We measured venoconstriction response to increasing concentration of exogenous NE. Our result shows an increase in vascular reactivity with HFD versus CD in the hypertensive groups (17- and 24-wk) while there was no difference in the pre-hypertensive group (10-wk) in males. This is in disagreement with previous report in Sprague Dawley male rats (Haddock and Hill, 2011). In contrast, vascular reactivity decreased with HFD compared to CD in hypertensive females while there was no difference in reactivity between diets from the prehypertensive group in females. We did not find previous data on females that either agrees or disagrees with our findings.

Since ATP is a co-transmitter that controls venous tone (Bobalova and Mutafova-Yambolieva, 2001), we measured venous response to exogenous ATP. Vascular reactivity to ATP is decreased with HFD compared to CD in both the pre-hypertensive and hypertensive females which is different from the data from NE reactivity.

MVs are surrounded and innervated by sympathetic nerves, and these nerves release NE and ATP (Bobalova and Mutafova-Yambolieva, 2001). We co-stained for markers of these transmitters and analyzed nerve densities. This was designed to give us a quantified measure of adrenergic and purinergic nerves. Our data show that TH-ir (NE marker) nerve fibers did not change with HFD compared to CD in males and females. Similarly, data from VNUT-ir (ATP marker) showed no effect of HFD on the nerve density in both sexes. However, these nerve densities do not necessarily reflect the amount released from the presynaptic terminal, and the venoconstriction that follows. Therefore, a direct measure of NE and ATP release via amperometry is warranted (Park et al., 2006).

NE content from MV provides us with an indirect measure of sympathetic nerve activity (Goldstein et al., 1983). We used HPLC to measure NE content in MV. Our findings show that NE decreases in tissue with respect with respect to age. Studies from DOCA-salt male rat reveal NE release is increased in MV (Luo et al., 2003; Luo et al., 2004). This is perhaps partly the reason for the depletion of NE from MV tissues (Luo et al., 2003), and for the increase in plasma NE in both sexes. However, it should be noted that NE released from the perivenous nerves will most of it (~95%) will be taken up by NET, and the rest will either be metabolized or diffuse away (Kreulen, 2003).

In conclusion, the present study demonstrated that HFD-induces equivalent hypertension in both sexes. α2-AR and NET are not responsible for this hypertension. Therefore, HFD may not affect the neurotransmission in the venous neuroeffector junction to cause hypertension in both sexes.

CHAPTER 6: CHARACTERIZING THE EFFECT OF HIGH FAT DIET ON THE NUMBER OF TH, VNUT AND COLOCALIZED IMMUNOSTAINS IN THE CELIAC GANGLION, MESENTERIC ARTERY, AND MESENTERIC VEIN FROM DAHL SALT-SENSITIVE RAT

1.0 Abstract

Cell bodies for the postganglionic sympathetic nerves are contained in the celiac ganglion (CG), and these nerves project out and innervate mesenteric artery (MA) and vein (MV). The effect of HFD on the types and numbers of these nerves is less known. We hypothesized that HFD increases the number of sympathetic nerves in the CG, MA and MV. We used male and female Dahl salt-sensitive rat on control (CD) and HFD. Rats were grouped into 10-, 17-, and 24-wk on diet before use. Immunohistochemical staining for TH, and VNUT were used followed by confocal imaging. Vesicular and nerve counting was performed using ImageJ. In CG, TH-immunoreactive (ir) vesicles were higher with HFD except in 10- and 17-wk males. VNUT-ir vesicles were higher with HFD in number in 17- and 24-wk females but lower in 10-wk males. Merge (colocalized)-ir vesicles were also higher with HFD in 17-wk females and 24-wk males. In MA, TH-ir was higher with HFD at 10-wk in both sexes. It was also higher in 24-wk males, but lower in 24-wk females. VNUT-ir nerves were higher with HFD in 10-wk female and 24-wk male and female rats. Merge-ir nerves were also higher in number in 24-wk males. In MV, TH-ir nerves were higher with HFD in 17-wk males, but lower in 24-wk females. VNUT-ir nerves were higher with HFD in 10-wk males, but lower with 10-wk females. These preliminary data show that HFD may alter the abundance of sympathetic neurotransmitter vesicles and nerves in the CG, MA and MV. In conclusion, the HFD has differential effect on the number of TH-, VNUT-, and co-localized- ir stains in the CG, MA and MV, but increasing the number of animals will be important to make any inference regarding these findings.

2.0 Introduction

Mesenteric arteries (MA) and veins (MV) are innervated by postganglionic sympathetic nerves projecting from celiac ganglia (CG) (Kreulen, 2003). NE and ATP are the two main neurotransmitters co-released from axonal sympathetic nerve terminals (varicosities) that regulate vascular tone and blood pressure (Burnstock and Ralevic, 1994; Sheng and Zhu, 2018). NE is synthesized in CG (Brokaw and Hansen, 1987) and varicosities (Thomas, 2011). Also, ATP is synthesized in CG (Ochoa-Cortes et al., 2010) and nerve terminal (Sperlagh and Vizi, 1996). NE and ATP are packaged in distinct vesicles although it is reported that they are co-stored in the same vesicle, too, in nerve terminal (Sperlagh and Vizi, 1996).

NE is stored in vesicular monoamine transporter 2 (VMAT2) whereas ATP is stored in VNUT within the same varicosity (Kaestner et al., 2019). Neurochemical characterization of CG from mice revealed the presence of VMAT2 in CG (Kaestner et al., 2019); however, there is no data showing localization of VNUT in CG. In addition, two separate populations of sympathetic axons have been suggested to innervate inferior MA and MV in the colon from female guinea pigs (Browning et al., 1999).

TH is a rate limiting enzyme in the synthesis of NE, and it is often used as a marker for NE (Weihe et al., 2006). Similarly, VNUT is used as a marker for ATP (Harada et al., 2018). The objective of the present study was to identify and quantify TH- and VNUT-ir nerves in the CG, MA, and MV. In addition, this study was intended to show colocalization of TH- and VNUT-ir nerves. These, then would allow us to better characterize the sympathetic neurotransmission in the mesenteric vasculature.

3.0 Results

TH and VNUT abundance in CG, MA, and MV in 10-wk rats. The number of TH- and VNUT-ir vesicles are lower in HFD compared to CD in CG from male rats (Fig. 33). Despite that, TH-ir in the periarterial nerves are more abundant in HFD compared to CD rats (Fig. 34) whereas VNUT-ir perivenous nerves are greater in number in HFD compared to CD rats (Fig. 35). In contrast, TH-ir vesicles are significantly higher in HFD compared to CD rat in CG from female rats (Fig. 36). Similar effect of the HFD is also observed in the periarterial TH-ir nerves in addition to greater VNUT-ir count in HFD vs. CD (Fig. 37). Nevertheless, VNUT-ir nerve count from veins is lower in HFD compared to CD (Fig. 38). Taken together, HFD (vs. CD) lowered the abundance of ganglionic TH- and VNUT-ir vesicles in males while elevating the number of TH-ir in females. In addition, HFD increased adrenergic nerves in both sexes, and increased purinergic nerves only in female mesenteric artery. Interestingly, HFD exhibited an opposing effect on the number of purinergic nerves in male and female mesenteric veins. Finally, colocalization of TH- and VNUT-ir vesicles and nerves is observed in CG, MA, and MV, but its abundance is more pronounced in the CG followed by MA. This is clearly shown in males, and to a lesser extent in females.

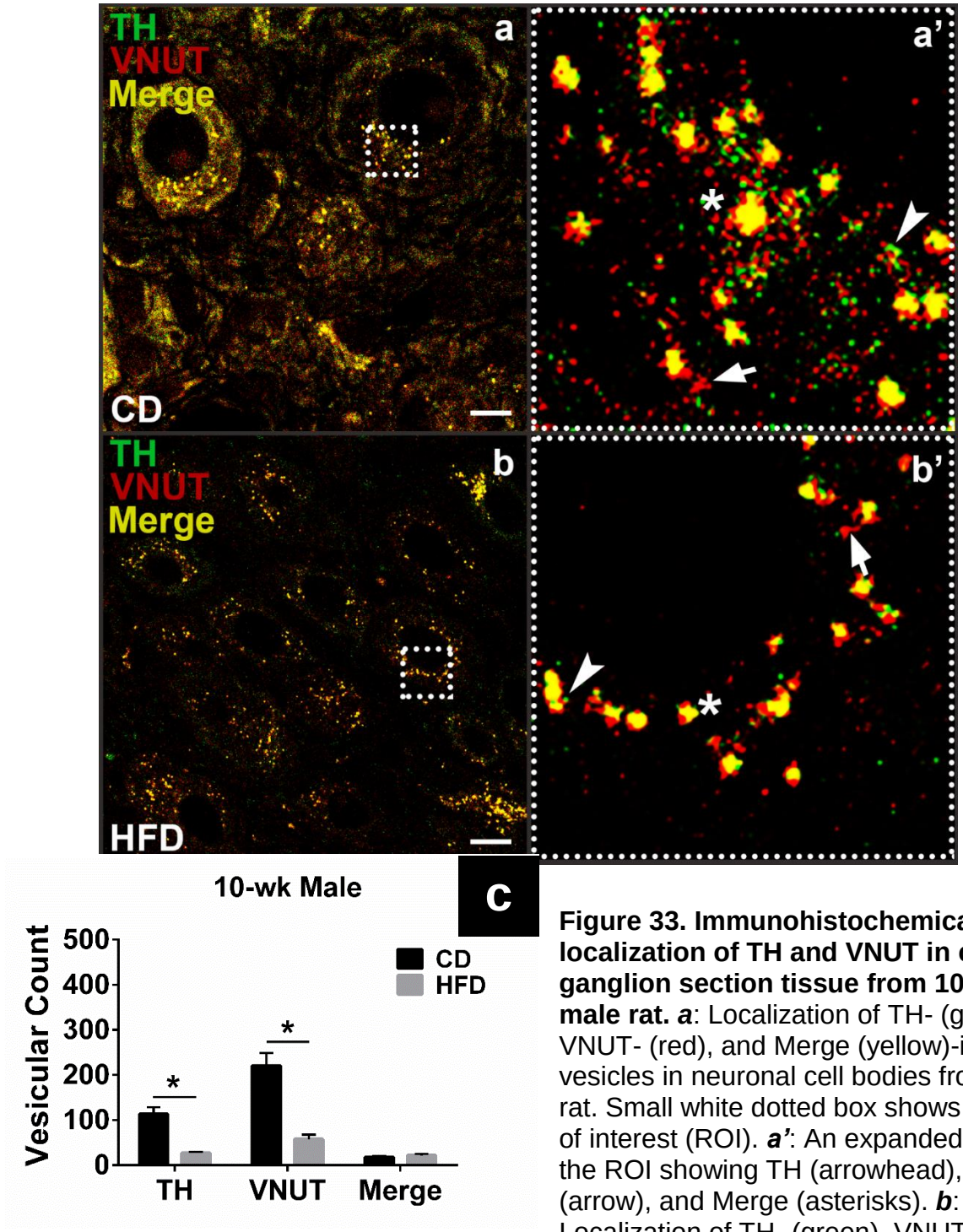
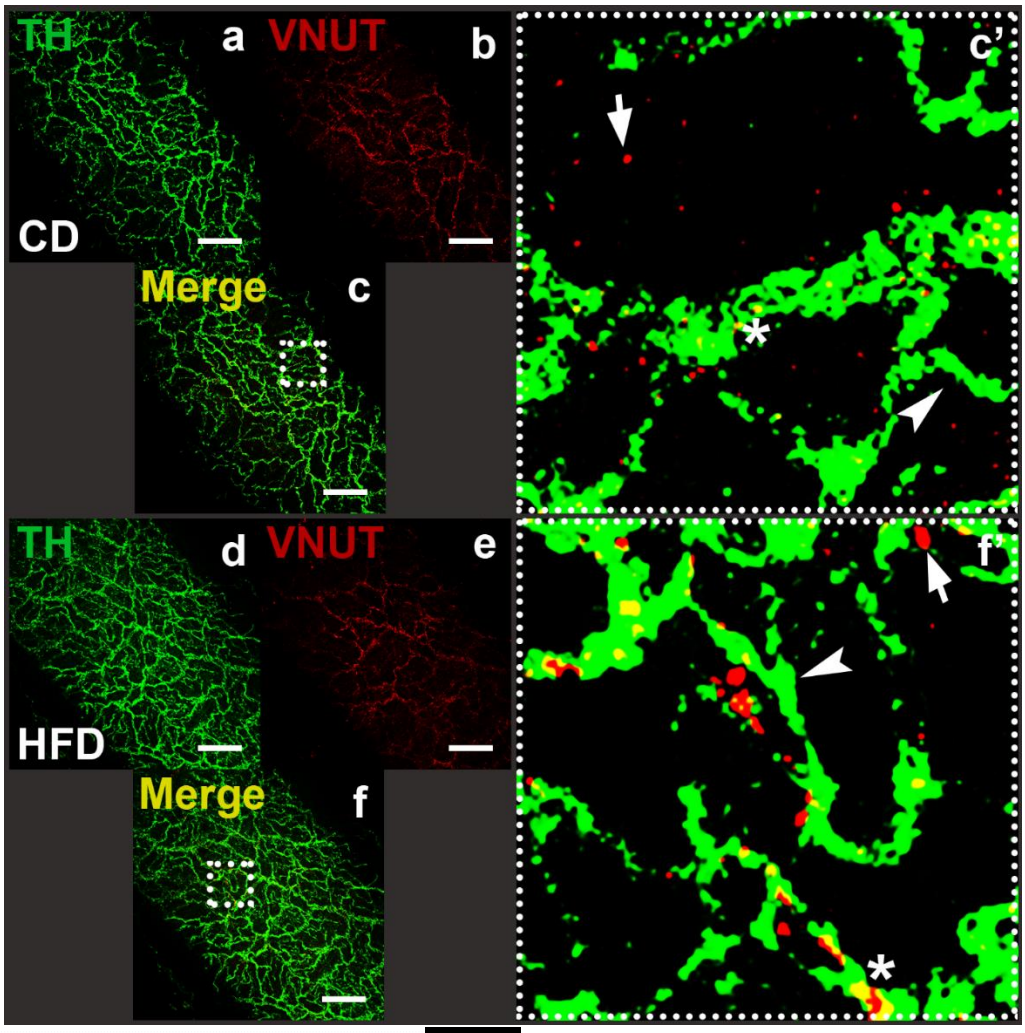


Figure 33. Immunohistochemical localization of TH and VNUT in celiac ganglion section tissue from 10-wk male rat. ***a***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from CD rat. Small white dotted box shows region of interest (ROI). ***a'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***b***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from HFD rat. Small white dotted box shows region of interest (ROI). ***b'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***c***: Histogram comparing vesicular count between CD and HFD in TH-, VNUT-, and Merge-ir vesicles. Numbers of TH-, and VNUT-ir vesicles are lower in HFD vs. CD. For ***a*** and ***b*** magnification is 120X and scale bar is 5 µm.



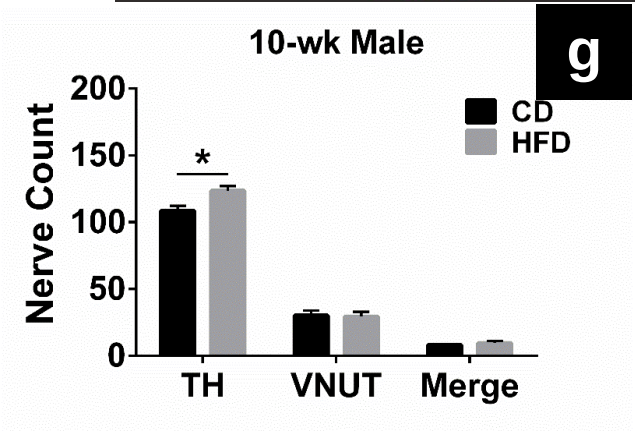
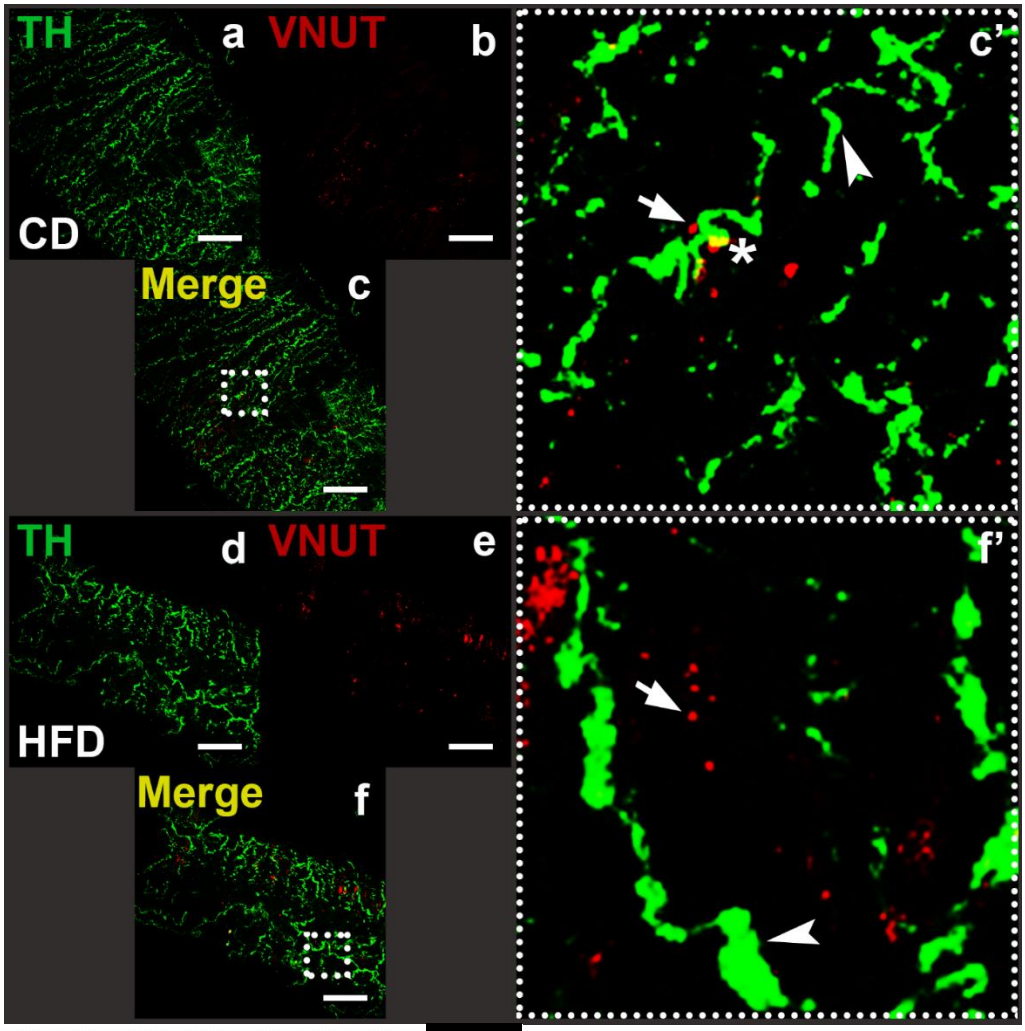


Figure 34. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric arteries from 10-wk male rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. Number of TH-ir nerves are higher in HFD vs. CD. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.



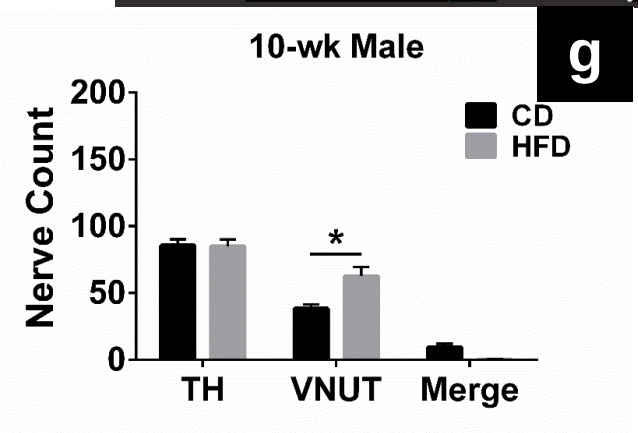
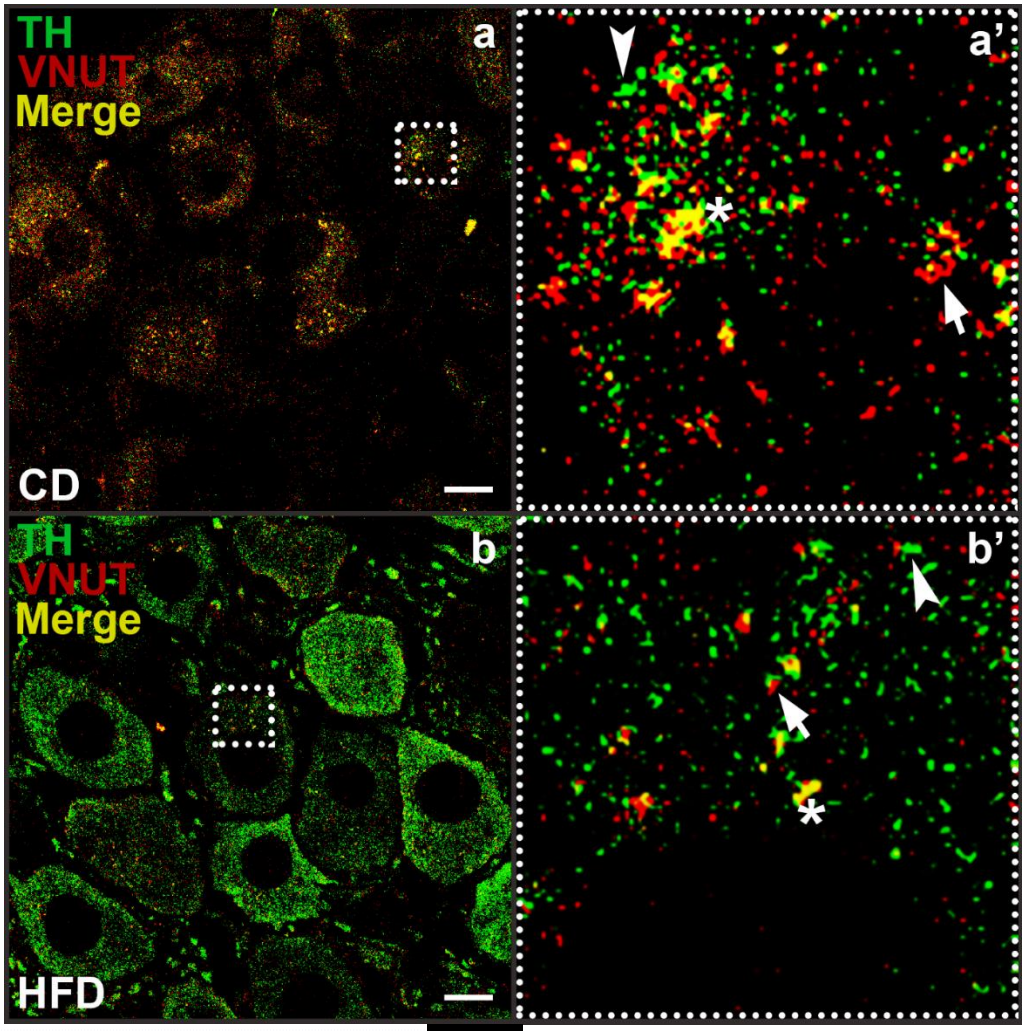


Figure 35. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric veins from 10-wk male rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in perivenous nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir perivenous nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. Number of VNUT-ir nerves are higher in HFD vs. CD. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.



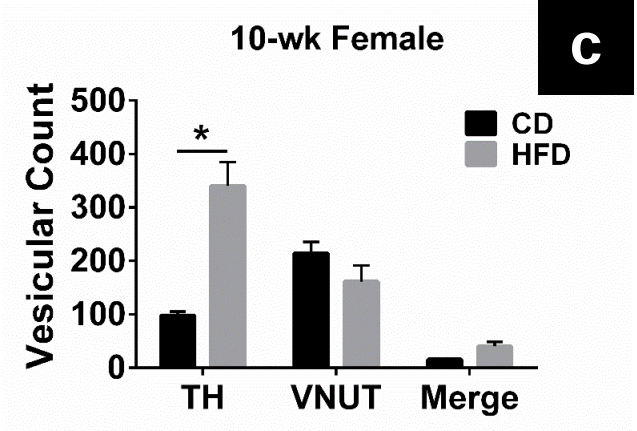


Figure 36. Immunohistochemical localization of TH and VNUT in celiac ganglion section tissue from 10-wk female rat. ***a***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from CD rat. Small white dotted box shows region of interest (ROI). ***a'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***b***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from HFD rat. Small white dotted box shows region of interest (ROI). ***b'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***c***: Histogram comparing vesicular count between CD and HFD in TH-, VNUT-, and Merge-ir vesicles. Numbers of TH-ir vesicles are higher in HFD vs. CD. For ***a*** and ***b*** magnification is 120X and scale bar is 5 µm.

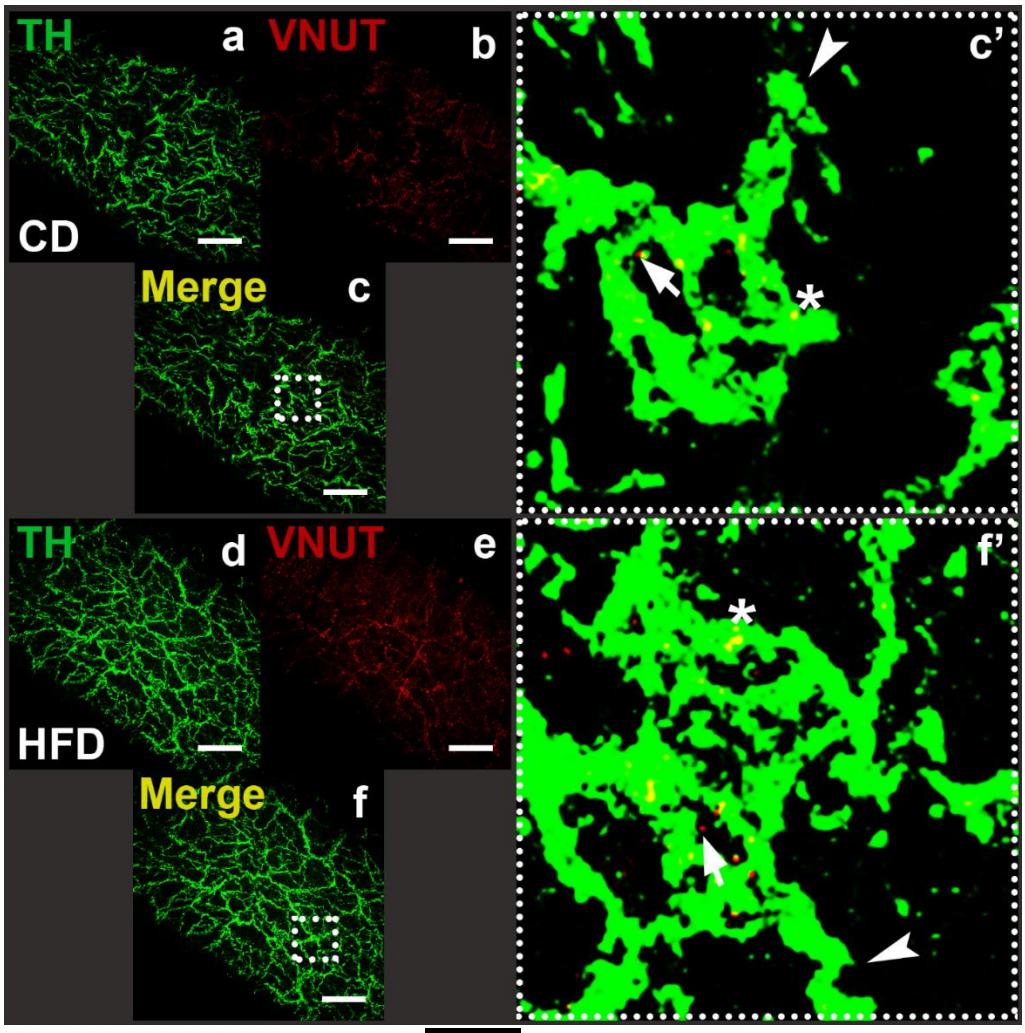
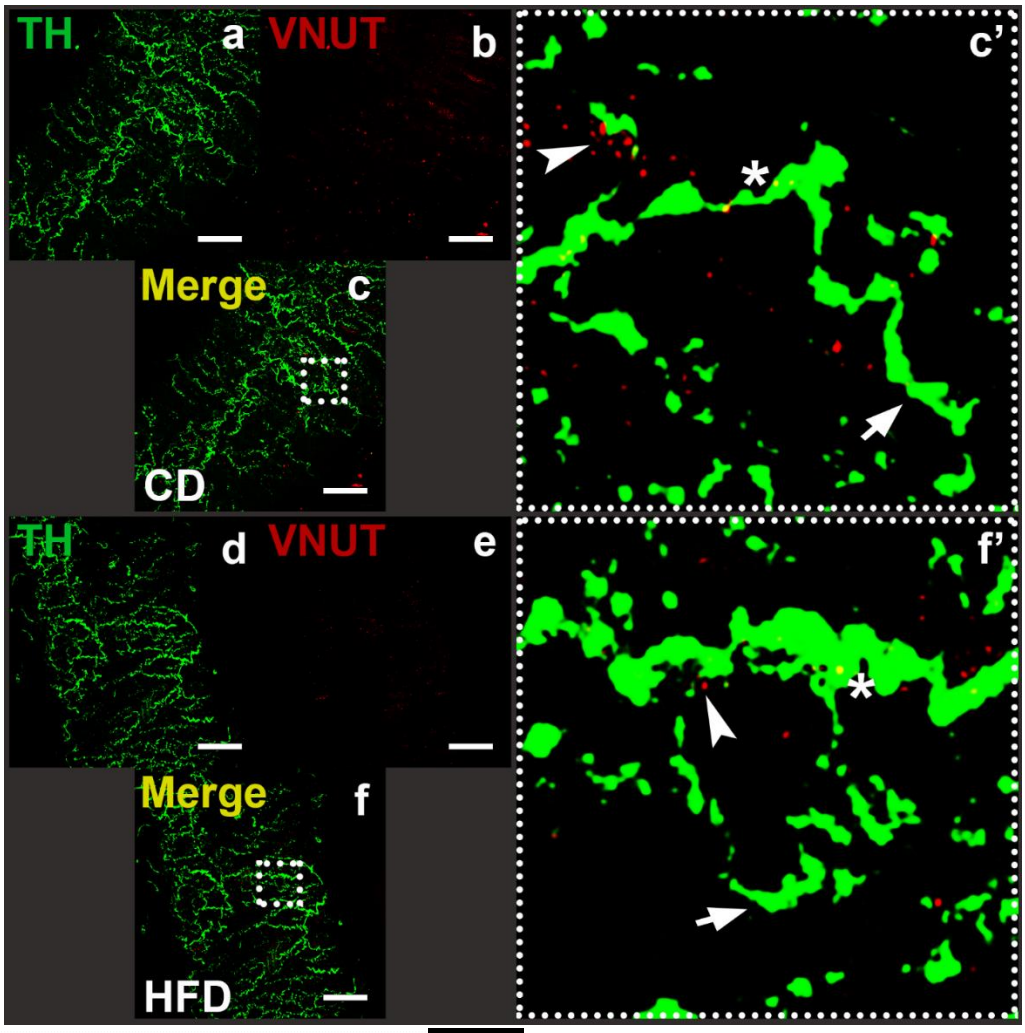


Figure 37. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric arteries from 10-wk female rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. Number of TH-, and VNUT-ir nerves are higher in HFD vs. CD. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.



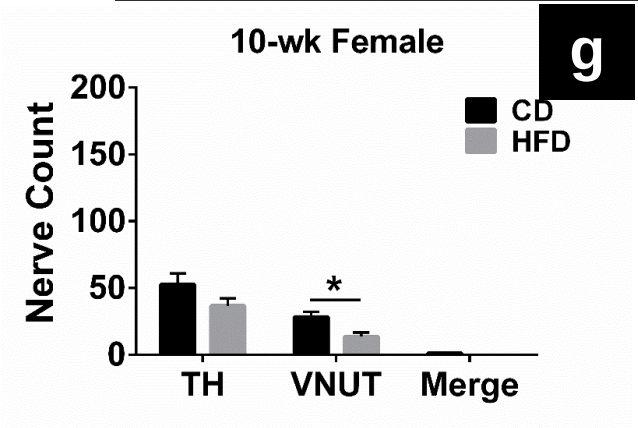
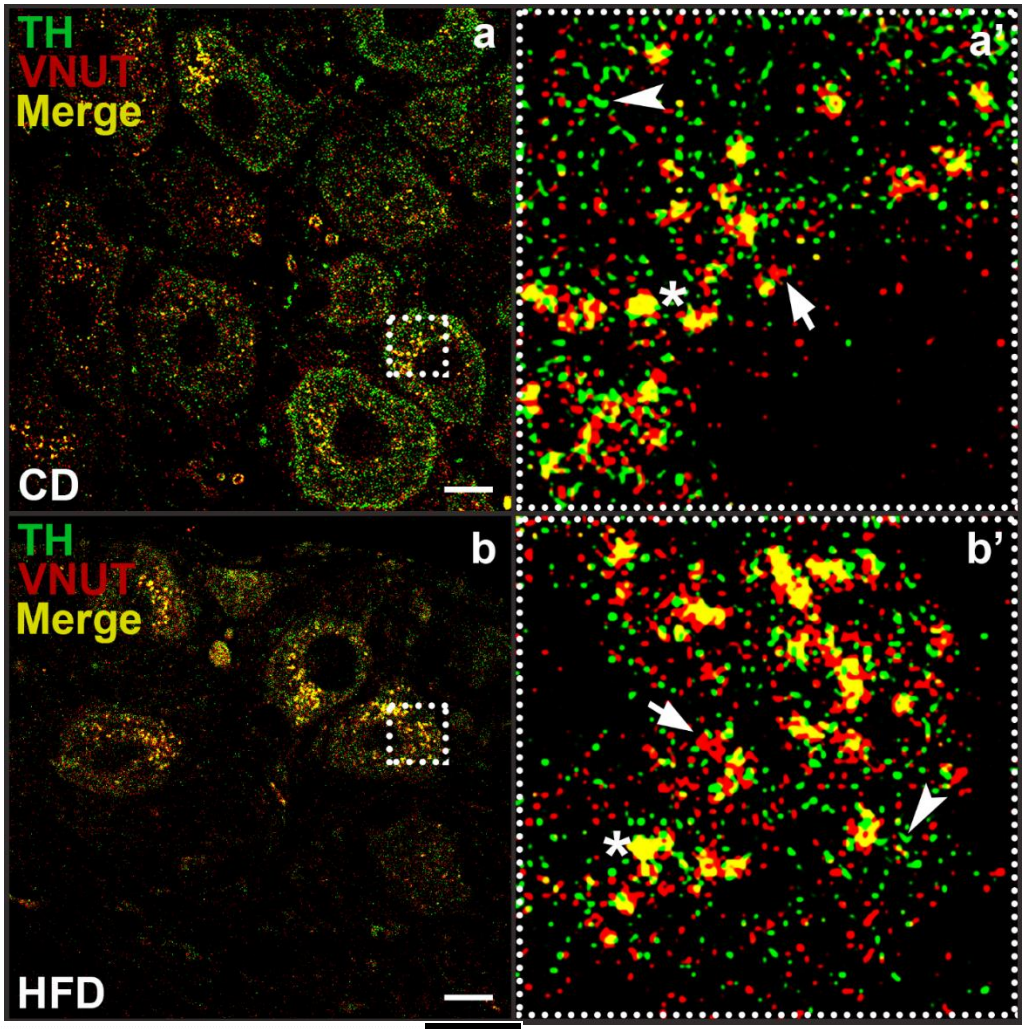


Figure 38. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric veins from 10-wk female rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. Number of VNUT-ir nerves are lower in HFD vs. CD. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.

TH and VNUT abundance in CG, MA, and MV in 17-wk rats. There is no HFD-induced change in the number of TH-, VNUT-, and Merge-ir vesicles in the CG from male rats (Fig. 39). Similarly, there is no difference in nerve density between HFD and CD rats in mesenteric artery; however, the adrenergic nerves are significantly more abundant compared to purinergic as well as colocalized nerve fibers regardless of diet (Fig. 40). Contrary to CG and MA, the number of TH-ir nerves in the mesenteric veins is greater in HFD compared to CD rats in males (Fig. 41). Unlike in CG from males, the number of TH-, VNUT-, and Merge-ir vesicles is higher in HFD compared to CD in females (Fig. 42). Although we observed diet-induced impact on the sympathetic distribution in the CG, that is not reflected on the perivascular nerves and hence there is no difference in the number of adrenergic and purinergic nerves between HFD and CD in the periarterial (Fig. 43) and perivenous (Fig. 44) nerves in females at 17-wk. It is noteworthy that the purinergic nerves surrounding the blood vessels are sparse at 17- compared to 10-wk. Taken together, sympathetic nerves are significantly reduced in number in the mesenteric vasculature at 17-wk in both sexes, and the HFD-related increase (vs. CD) in the three types of sympathetic populations in CG from females does not correlate with the absence of similar changes in the vasculature.



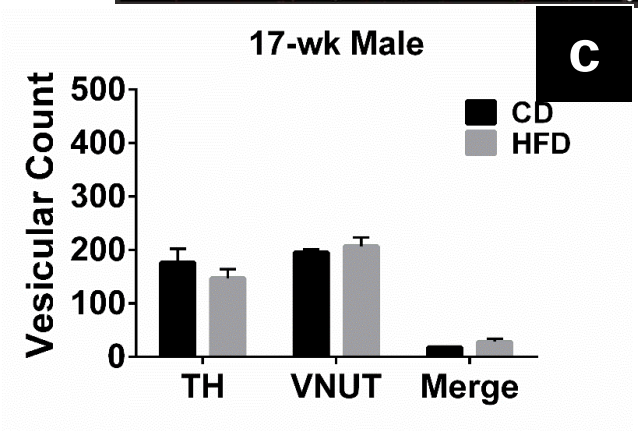
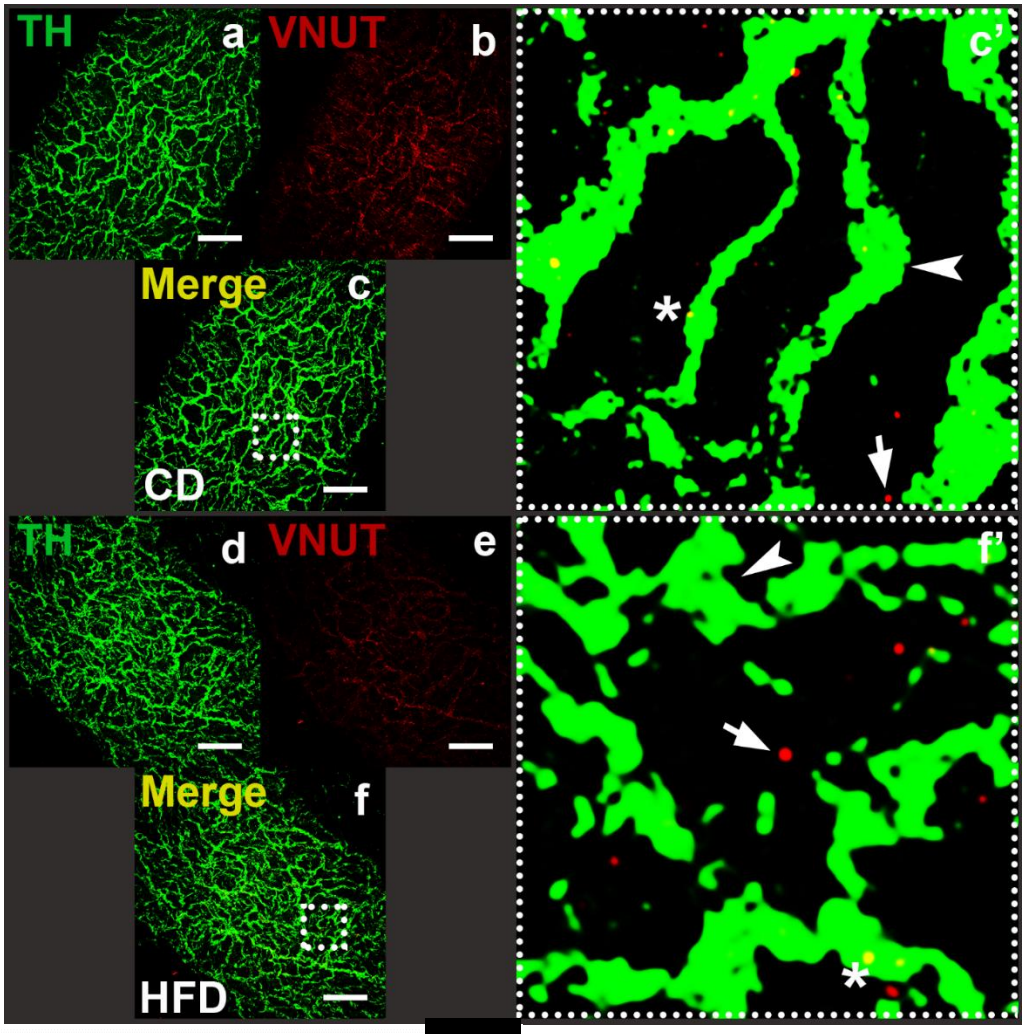


Figure 39. Immunohistochemical localization of TH and VNUT in celiac ganglion section tissue from 17-wk male rat. ***a***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from CD rat. Small white dotted box shows region of interest (ROI). ***a'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***b***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from HFD rat. Small white dotted box shows region of interest (ROI). ***b'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***c***: Histogram comparing vesicular count between CD and HFD in TH-, VNUT-, and Merge-ir vesicles. For ***a*** and ***b*** magnification is 120X and scale bar is 5 µm.



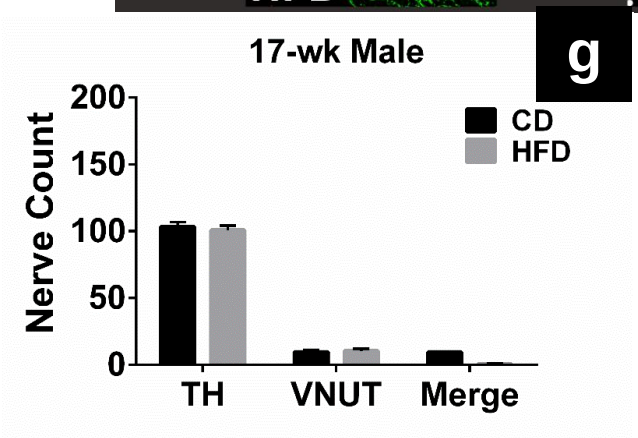
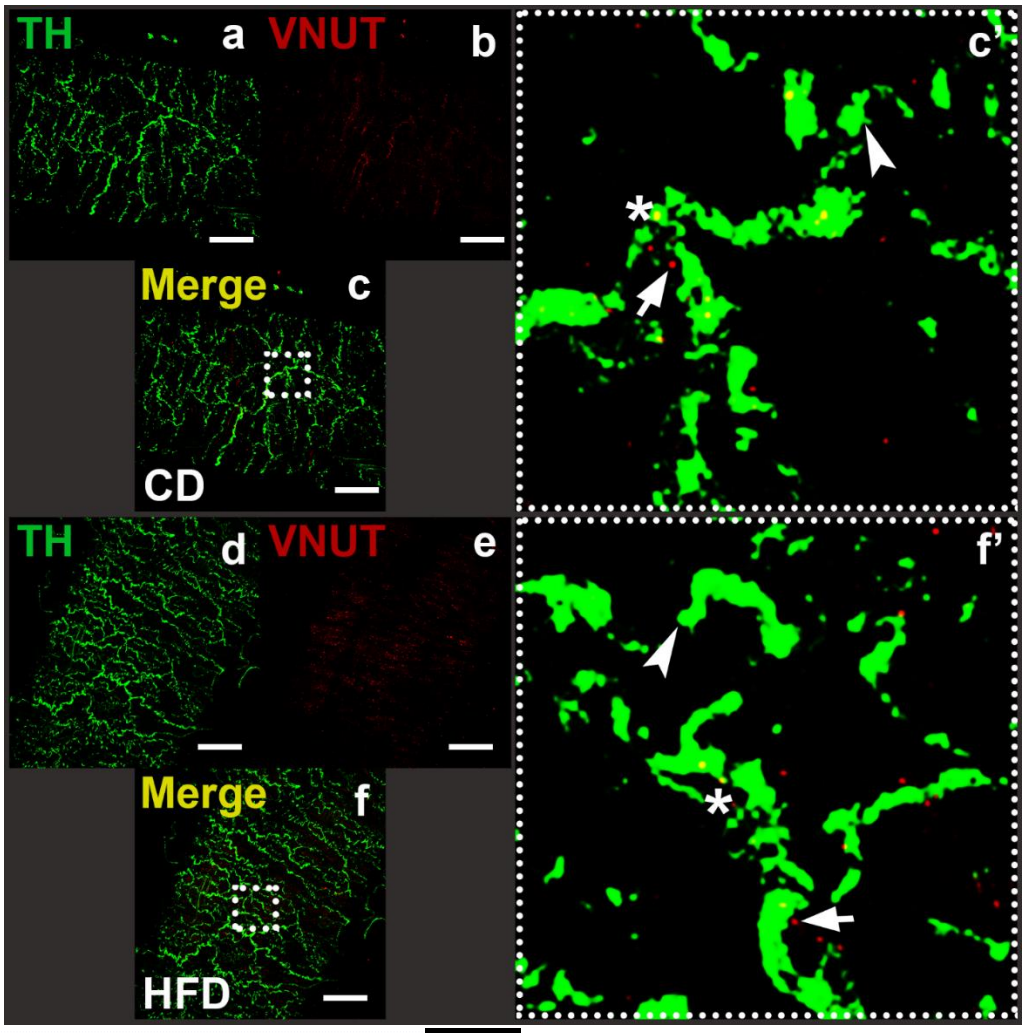


Figure 40. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric arteries from 17-wk male rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.



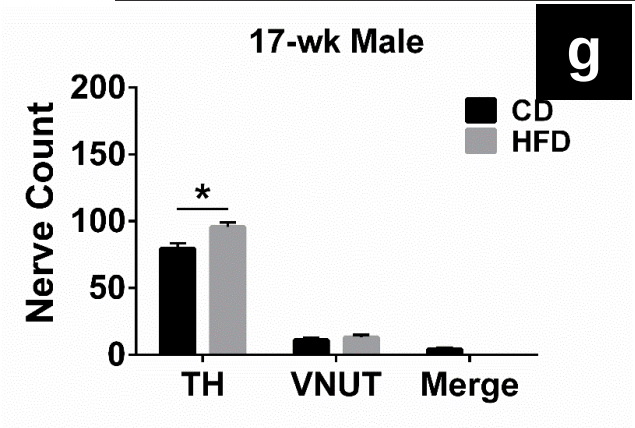


Figure 41. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric veins from 17-wk male rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. Number of TH-ir nerves are higher in HFD vs. CD. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.

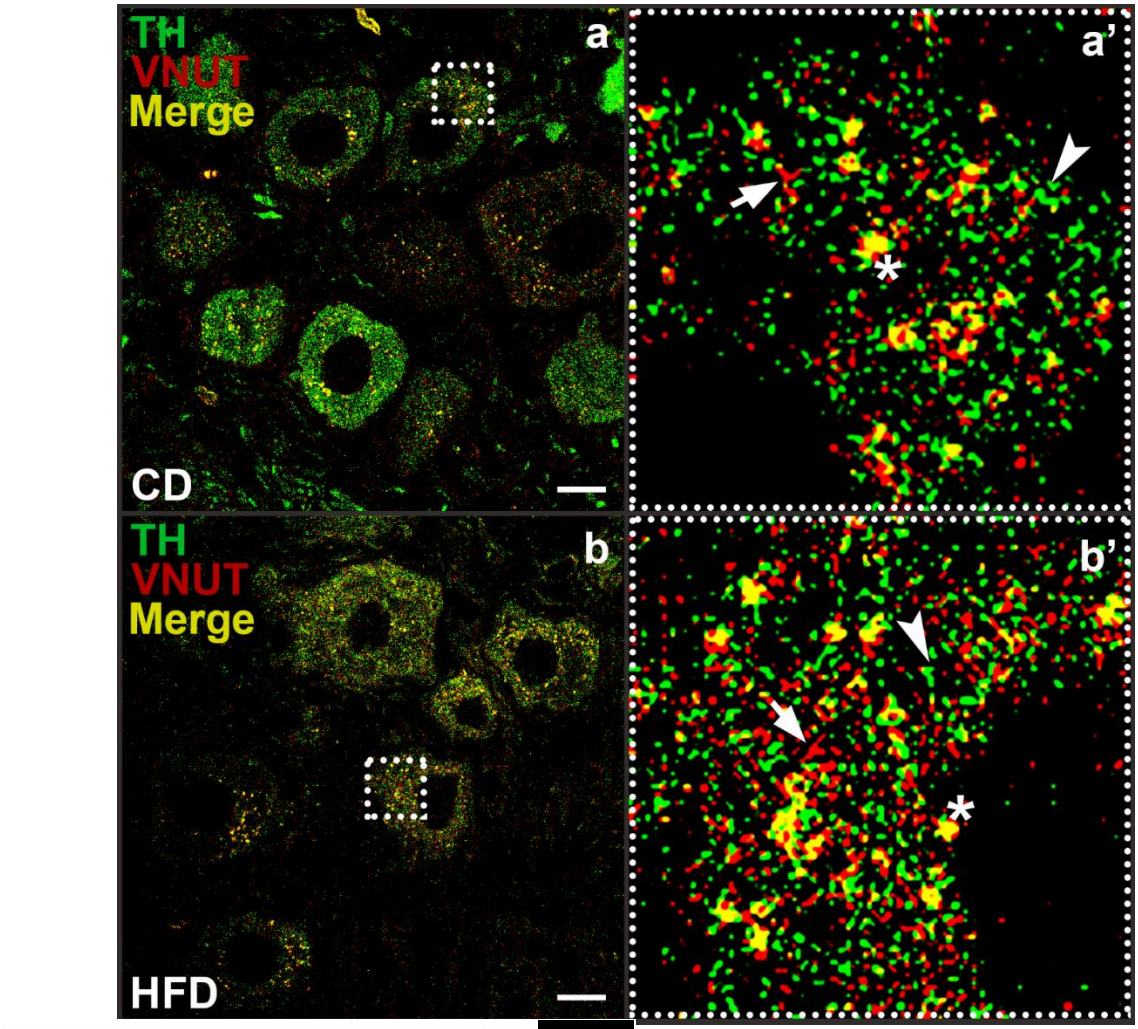
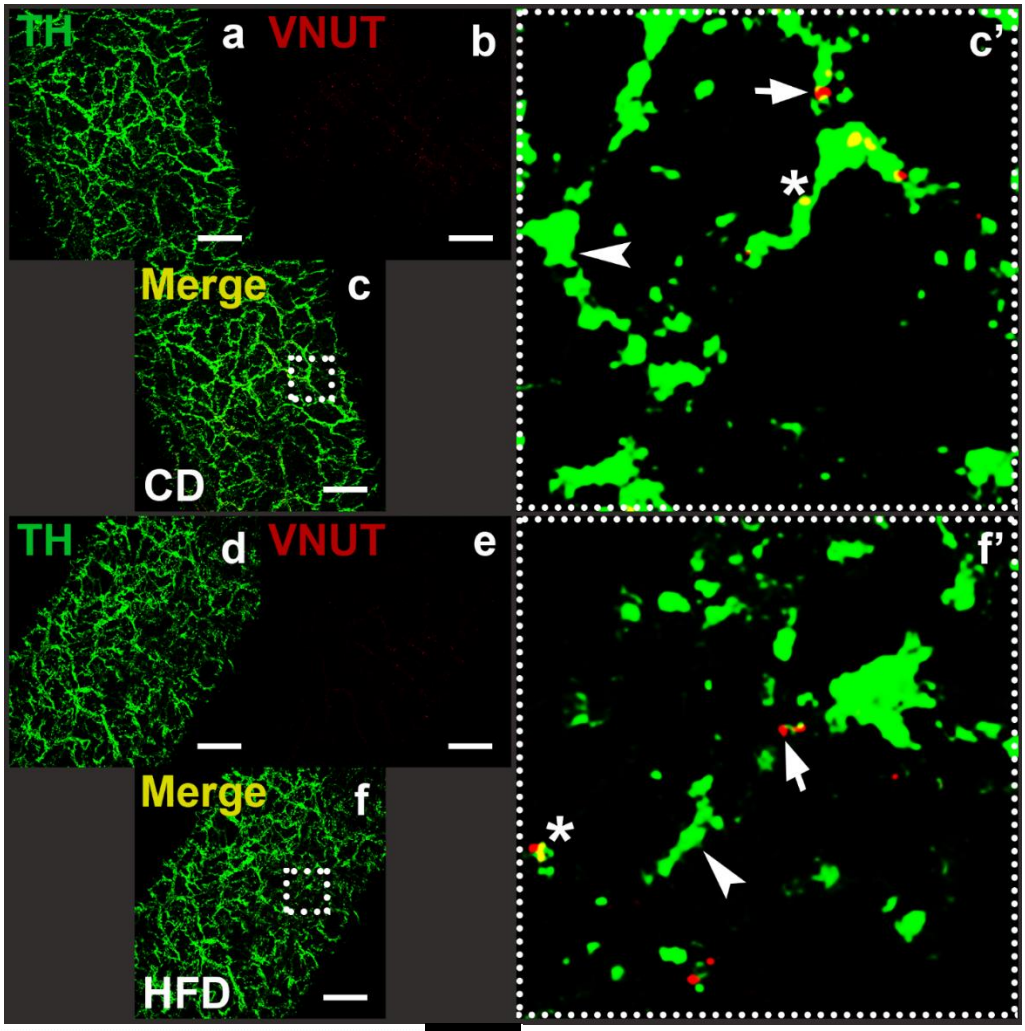


Figure 42. Immunohistochemical localization of TH and VNUT in celiac ganglion section tissue from 17-wk female rat. ***a***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from CD rat. Small white dotted box shows region of interest (ROI). ***a'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***b***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from HFD rat. Small white dotted box shows region of interest (ROI). ***b'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***c***: Histogram comparing vesicular count between CD and HFD in TH-, VNUT-, and Merge-ir vesicles. Numbers of vesicles in TH-, VNUT-, and Merge-ir are higher in HFD vs. CD. For ***a*** and ***b*** magnification is 120X and scale bar is 5 µm.



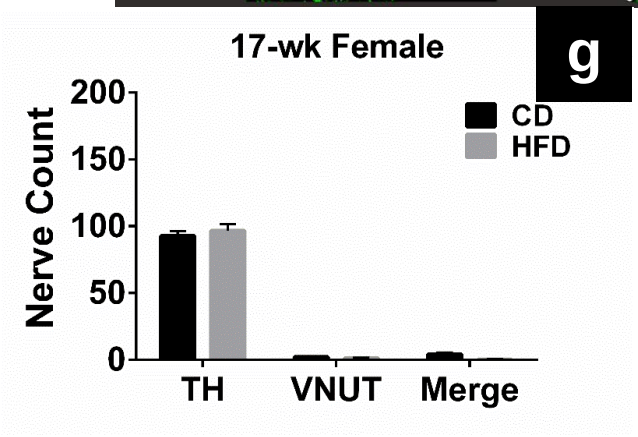
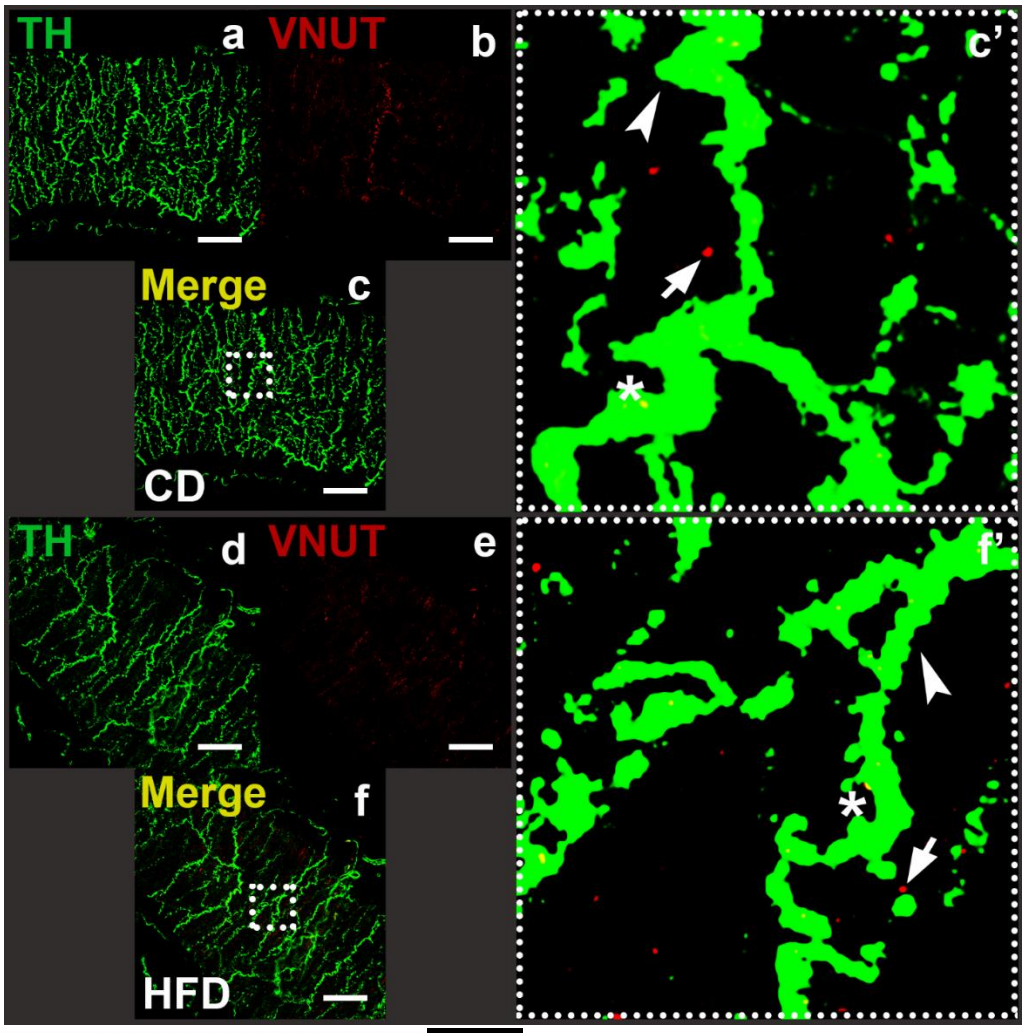


Figure 43. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric arteries from 17-wk female rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.



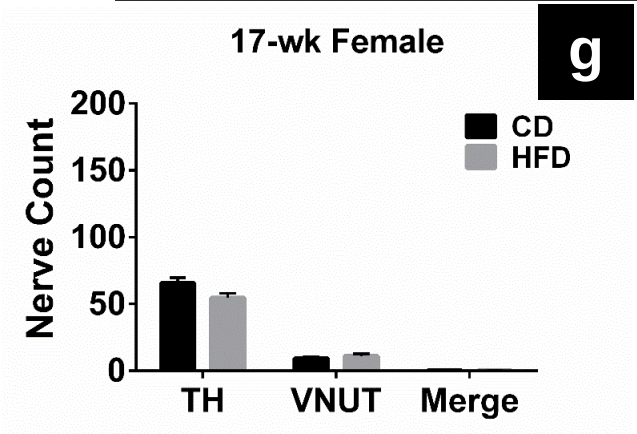


Figure 44. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric veins from 17-wk female rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.

TH and VNUT abundance in CG, MA, and MV in 24-wk rats. The number of vesicular counts for TH-, and Merge-ir are higher in HFD compared to CD rats in males. Moreover, the vesicular count for VNUT-ir is lower in HFD versus CD rats although not statistically significant (Fig. 45). The data from CG is reflected in the MA (except in VNUT) in that the nerve count for TH-, VNUT-, and Merge-ir are greater in HFD compared to CD (Fig. 46). In contrast, there is no change in nerve count in the mesenteric veins in addition to the sparse presence of purinergic nerves (Fig. 47). In females, vesicular count of TH-, and VNUT-ir are higher in HFD compared to CD. Vesicular count of Merge-ir in HFD is also greater compared to CD although not significantly (Fig. 48). Periarterial nerve count is lower for TH-ir in HFD compared to CD, but higher for VNUT-ir in HFD versus CD (Fig. 49). Consistent with MA, the nerve count for TH-ir is lower in HFD compared to CD in mesenteric vein (Fig. 50). Overall, these data show that HFD-induced elevation in sympathetic innervation is predominantly purinergic in females while it involves both purinergic and adrenergic in males from mesenteric arteries. Moreover, there is no change in perivenous nerve count in males, but an adrenergic reduction in females similar to in the arteries.

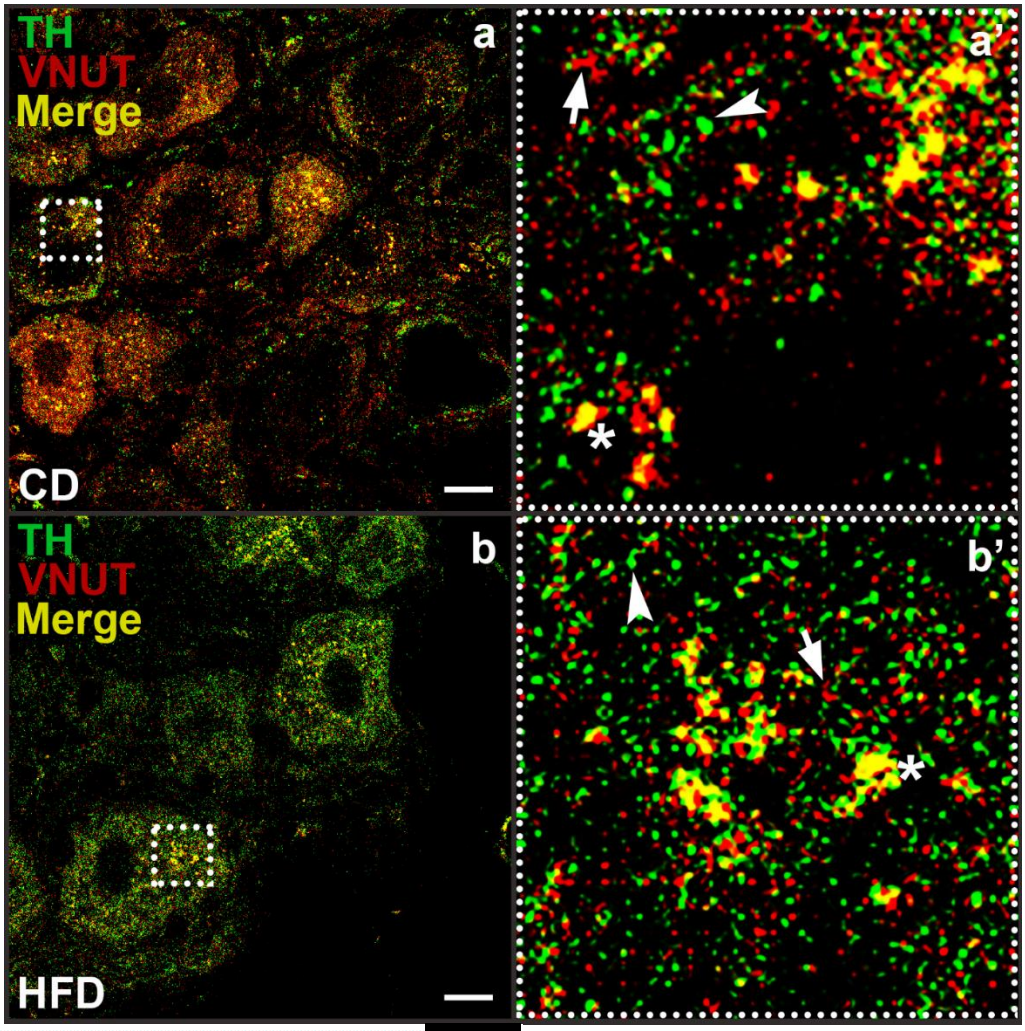
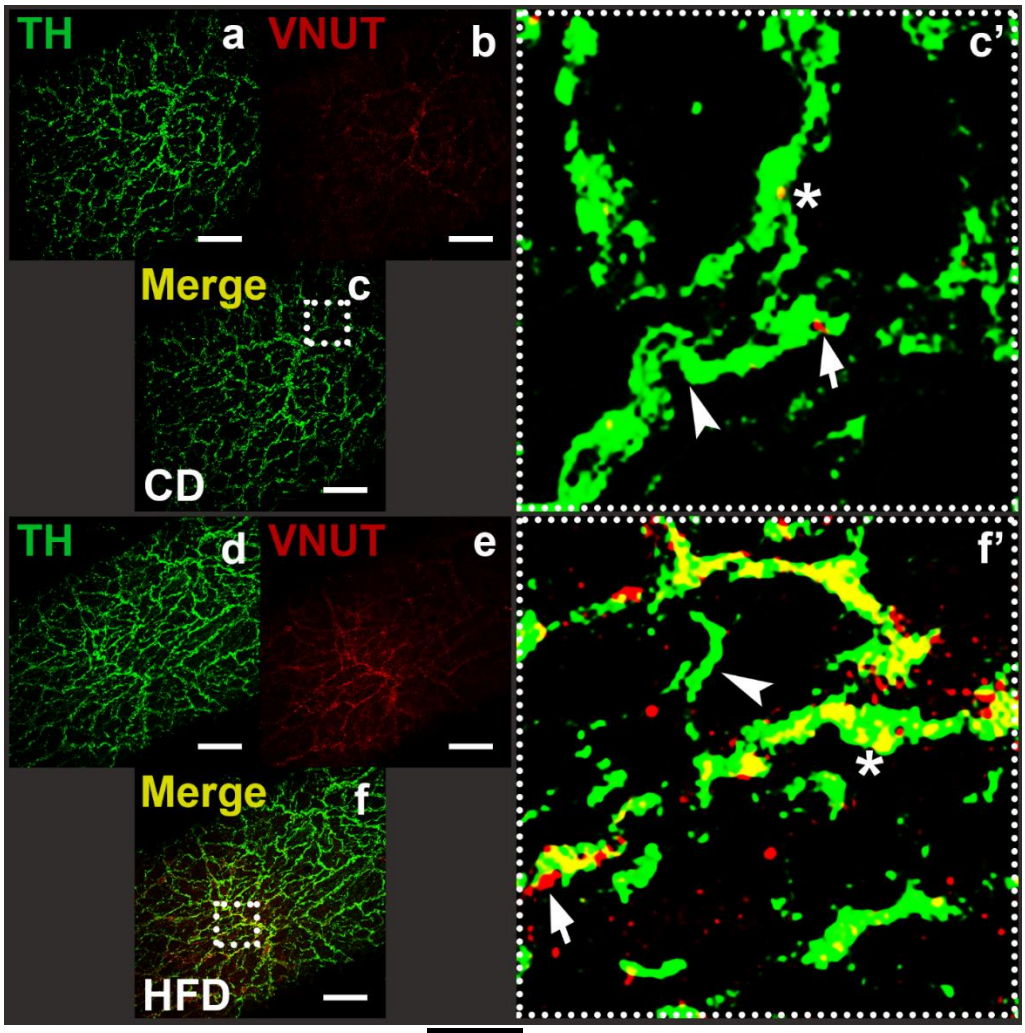


Figure 45. Immunohistochemical localization of TH and VNUT in celiac ganglion section tissue from 24-wk male rat. ***a***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from CD rat. Small white dotted box shows region of interest (ROI). ***a'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***b***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from HFD rat. Small white dotted box shows region of interest (ROI). ***b'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***c***: Histogram comparing vesicular count between CD and HFD in TH-, VNUT-, and Merge-ir vesicles. Numbers of vesicles in TH-, and Merge-ir are higher in HFD vs. CD. For ***a*** and ***b*** magnification is 120X and scale bar is 5 µm.



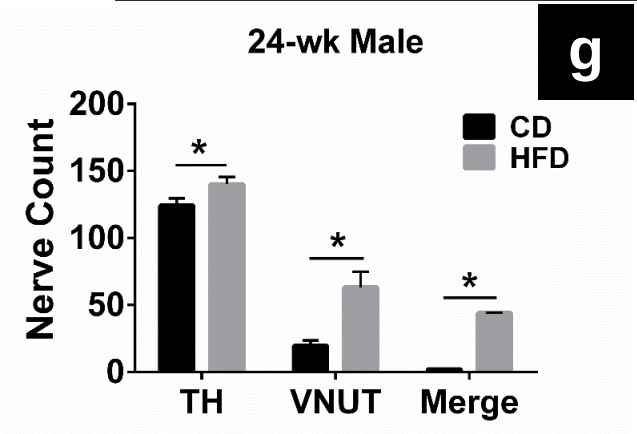
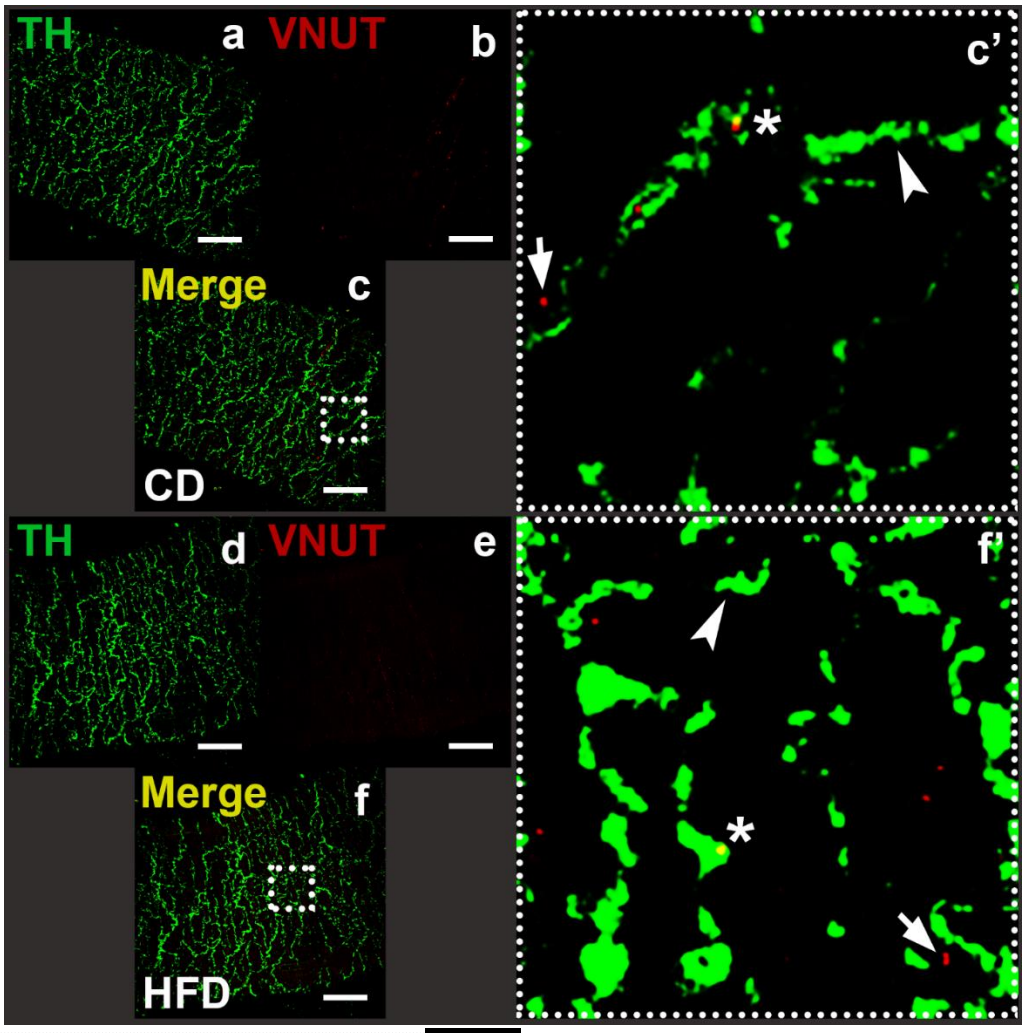


Figure 46. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric arteries from 24-wk male rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. Numbers of vesicles in TH-, VNUT- and Merge-ir are higher in HFD vs. CD. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.



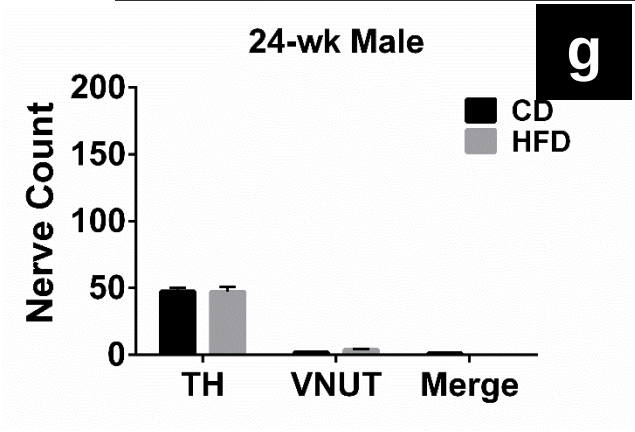
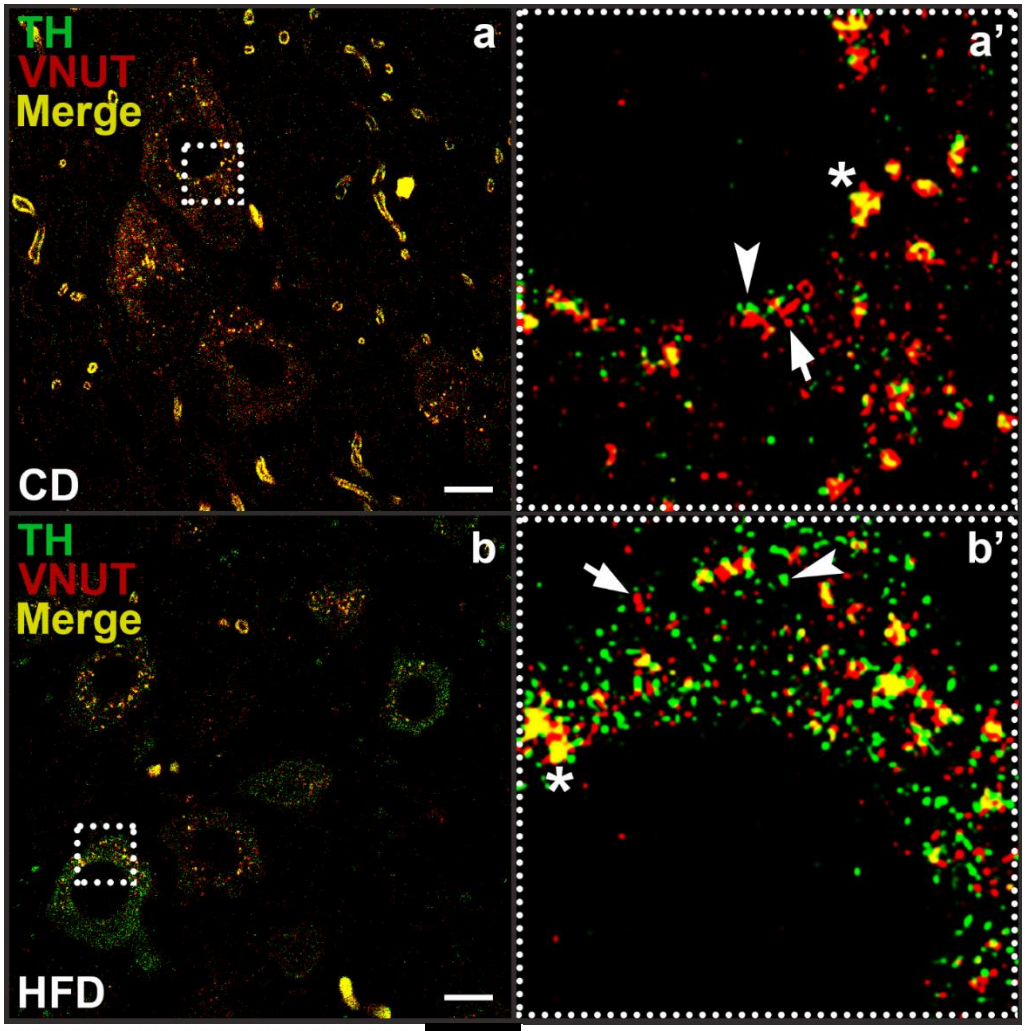


Figure 47. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric veins from 24-wk male rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.



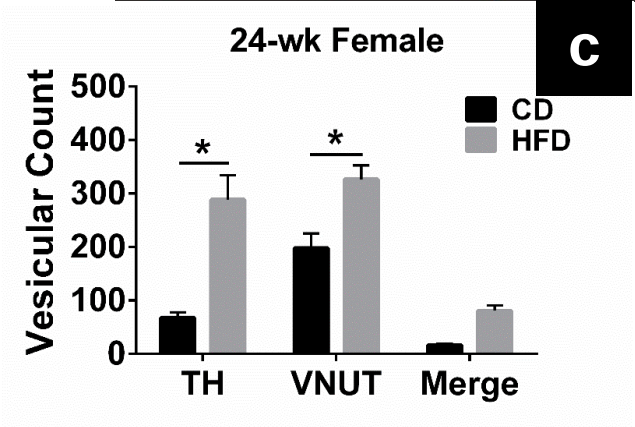
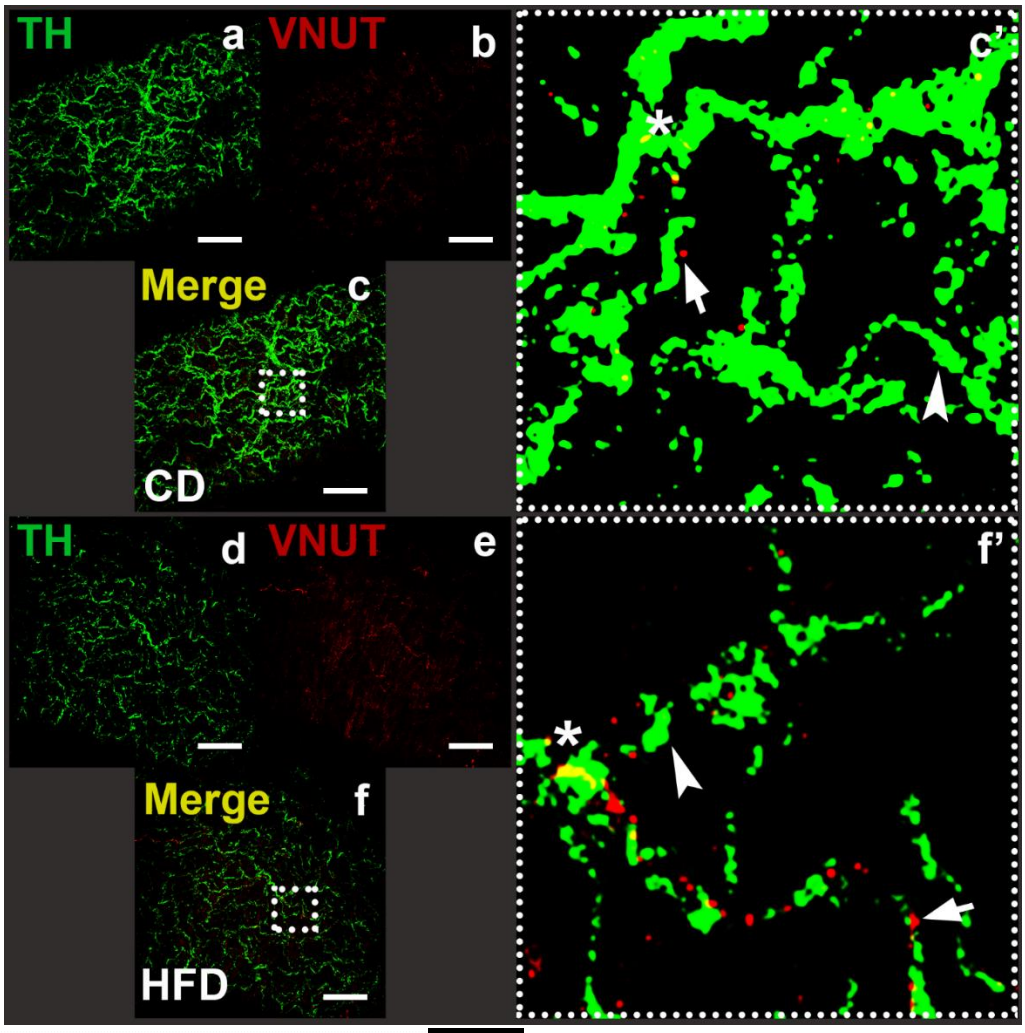


Figure 48. Immunohistochemical localization of TH and VNUT in celiac ganglion section tissue from 24-wk female rat. ***a***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from CD rat. Small white dotted box shows region of interest (ROI). ***a'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***b***: Localization of TH- (green), VNUT- (red), and Merge (yellow)-ir vesicles in neuronal cell bodies from HFD rat. Small white dotted box shows region of interest (ROI). ***b'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). ***c***: Histogram comparing vesicular count between CD and HFD in TH-, VNUT-, and Merge-ir vesicles. Numbers of vesicles in TH-, and VNUT-ir are higher in HFD vs. CD. For ***a*** and ***b*** magnification is 120X and scale bar is 5 µm.



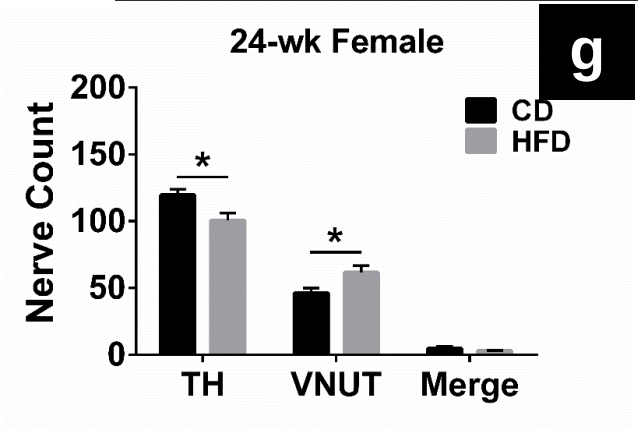
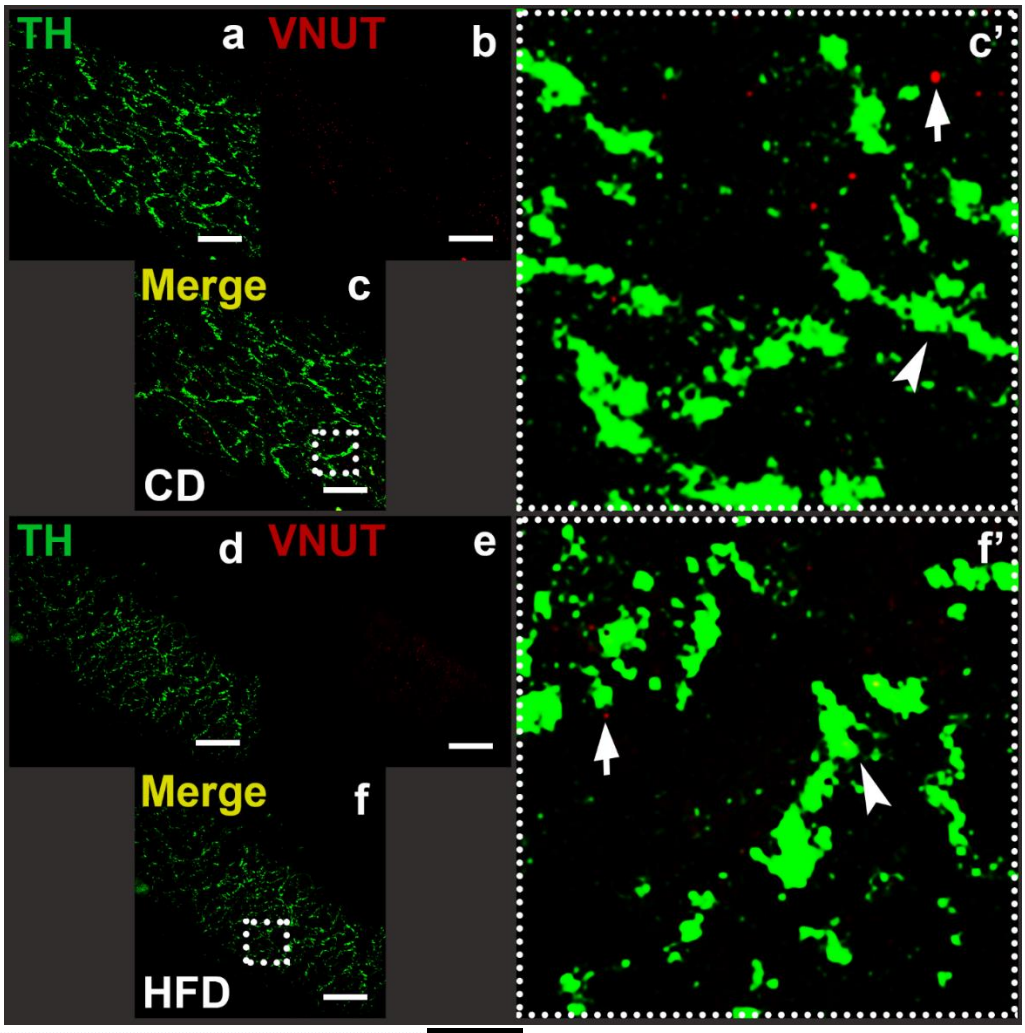


Figure 49. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric arteries from 24-wk female rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. Numbers of vesicles in TH-ir are lower in HFD vs. CD whereas they are higher in VNUT-ir in HFD vs. CD. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.



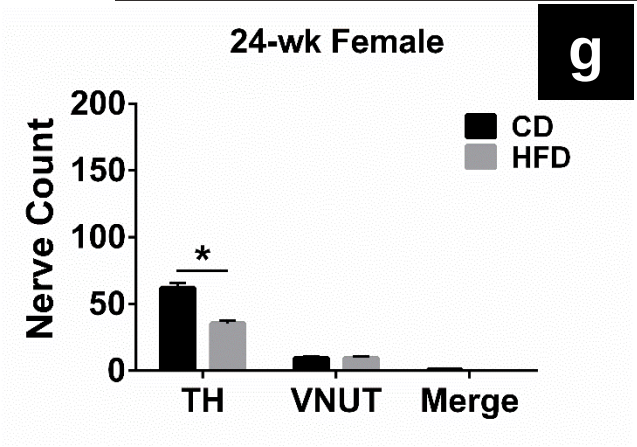


Figure 50. Immunohistochemical localization of TH and VNUT in whole mount preparations of mesenteric veins from 24-wk female rat. Localization of TH- (***a***, green), VNUT- (***b***, red), and Merge (***c***, yellow)-ir in periarterial nerve fibers from CD rat. Small white dotted box (***c***) shows ROI. ***c'***: An expanded view of the ROI showing TH (arrowhead), VNUT (arrow), and Merge (asterisks). Localization of TH- (***d***, green), VNUT- (***e***, red), and Merge (***f***, yellow)-ir periarterial nerve fibers from HFD rat. Small white dotted box (***f***) shows ROI. ***f'***: An expanded view of the ROI showing TH-, VNUT-, and Merged-ir nerves. ***g***: Histogram comparing nerve count between CD and HFD in TH-, VNUT-, and Merge-ir nerves. Numbers of vesicles in TH-ir are lower in HFD vs. CD. For all images except ***c'*** and ***f'*** magnification is 40X and scale bar is 25 µm.

Table 8. Summary of data for figures 33-50. The arrows show higher (up) or lower (down) levels in vesicular and nerve counts when comparing HFD to CD.

		10-wk		17-wk		24-wk	
		Male	Female	Male	Female	Male	Female
CG	TH	↓	↑		↑	↑	↑
	VNUT	↓			↑		↑
	Merge				↑	↑	
MA	TH	↑	↑			↑	↓
	VNUT		↑			↑	↑
	Merge					↑	
MV	TH			↑			↓
	VNUT	↑	↓				
	Merge						

As shown in the above table 8, the data from this part of the study shows: 1) there are three subpopulations of sympathetic vesicles in the CG; 2) there are three subpopulations of sympathetic nerves in the MA; 3) there are mainly TH-ir nerves in the MV with a fewer to nothing in VNUT-, and Merge-ir localizations in MV; and 4) the distribution of sympathetic nerves in the CG is not necessarily reflected in the perivascular nerves in the mesenteric blood vessels.

4.0 Discussion

The present study was intended to test the hypothesis that HFD induces higher level of TH- and VNUT-ir staining in the CG and mesenteric vasculature. These are our main findings: 1) there are three distinct groups of sympathetic markers in the CG, MA, and MV; 2) HFD may induce upregulation of TH and VNUT but in few instances it downregulates such expression; and 3) the number of TH- and VNUT-ir nerves and varicosities is not consistently similar between CG and mesenteric vasculature.

This study found that there are three subpopulations of sympathetic nerves in CG, MA, and MV, namely TH-ir, VNUT-ir, and colocalized nerves. Although retrogradely-labelled neuropeptide Y (NPY)-stained nerves have been previously reported from inferior celiac ganglion in female guinea pigs (Browning et al., 1999), and TH, NPY and VMAT2 staining from male and female mice from sympathetic nerves that project to the spleen and stomach (Kaestner et al., 2019), this study is the first to identify the three subpopulations by co-labelling for TH and VNUT in CG, MA and MV. Moreover, this was demonstrated in male and female Dahl ss rats of three age groups. The study from the Kaestner group demonstrated that the neurons in the celiac ganglion are heterogenous in that some contain TH, yet others do not. It is known that ATP is present in CG neurons as an energy currency and a neurotransmitter (Ochoa-Cortes et al., 2010). Moreover, it is not a surprise that TH-ir neurons are present in CG as was acknowledged from guinea-pig (Macrae et al., 1986) and porcine (Palus and Calka, 2016) studies. Therefore, our finding corroborates these previous studies.

The proportion of adrenergic and purinergic contraction is known to depend on the frequency of stimulation. It means that adrenergic constriction is dominant at higher

frequency whereas purinergic constriction is dominant at lower frequency (Muramatsu et al., 1989; Haddock and Hill, 2011). This alternating pattern is related to frequency-dependent vesicular release of NE and ATP. Despite differences in the release mechanisms, our data unequivocally shows that TH-ir nerves are far greater in number than VNUT-ir nerves in both sexes and at all ages from MA and MV. This indicates that at the physiologically relevant frequency (~5Hz)(Kenney, 1994) required for the release of NE and ATP, it is highly likely that more NE storage will be exocytosed into the neuroeffector junction. It also implies that NE-mediated regulation of arterial tone is predominant. Additionally, adrenergic transmission is dominant in MV (Park et al., 2007). This is consistent with our data and clearly shown by the significant level of TH-ir compared to VNUT-ir nerves in both sexes and at all ages. Like in MA, TH-ir was higher than VNUT-ir implying that adrenergic nerves are the main drivers in maintaining venous tone (Perez-Rivera et al., 2005; Park et al., 2007).

We report here that HFD alters the abundance of vesicular contents in CG, and sympathetic nerves in the mesenteric vasculature. Our data from CG and MV are novel and similar studies with the sympathetic targets has not been shown before. This trend started at 17-wk in females while males began to show similar changes at 24-wk. Hence, HFD may affect females early on although these female rats do not become obese. The high level of TH-ir in MV from 17-wk males is difficult to interpret since there is no change in MA and CG to compare it to. The lower level of TH-ir in MV from 24-wk female may be a countermeasure to the high-level TH-ir in CG at the same time point. Alternatively, the lower level of TH-ir in HFD in MV and MA at 24-wk in females may be to counter an elevated sympathetic activity from other regions, such as the kidney (da

Silva et al., 2009; Kalil and Haynes, 2012). In MA, TH-ir is higher in HFD compared to CD from 24-wk male. This is consistent with a previous study showing increase in synaptophysin and catecholamine immunostaining in obese male 24-wk Sprague Dawley (Haddock and Hill, 2011).

We anticipated to observe that there would be similar effect of the HFD on the abundance of the TH-, VNUT-, and Merge-ir sympathetic populations in CG, MA, and MV. The only consistent effect of HFD was between CG and MA on TH-ir nerve at 10-wk in females, and on TH- and Merge-ir nerves at 24-wk in males. One reason as to why we didn't see such consistency in all groups may be due to the fact that neurons in the celiac ganglion are not exclusively projecting out to the mesenteric blood vessels. Some of the postganglionic nerves also innervate other abdominal organs, such as the liver, stomach, and pancreas (Li et al., 2010).

In conclusion, our preliminary data provides insight into the type and number of TH- and VNUT-ir vesicle/nerves in the CG, MA, and MV. Quantification of TH, VNUT and colocalized nerves in these locations broaden our understanding of the peripheral sympathetic nervous system in the mesenteric bed. In doing so, these findings can be used as a foundation for future studies to further understand the role of sympathetic nerves in hypertension.

CHAPTER 7: SUMMARIES, PERSPECTIVES, AND FUTURE DIRECTIONS

1.0 General Summary, Discussion, and Overall Conclusion

Autonomic regulation of blood pressure is partly achieved by the postganglionic sympathetic nerves innervating mesenteric arteries and veins. The prejunctional nerves release NE, ATP, and NPY to maintain physiologically desired vascular tone. NE is known to play the dominant role in such function. Therefore, the release, and metabolism of NE is highly regulated in the neuroeffector junction. Release of NE is controlled by the negative feedback inhibition mediated by α2-AR which is localized in the cell membrane of the presynaptic nerve terminal. Previous studies from DOCA-salt hypertension have demonstrated that this receptor is impaired leading to uncontrolled release of NE. Further, the availability of NE in the neuroeffector junction is monitored by a NE reuptake mechanism which is facilitated by NET that is localized in the presynaptic nerve. In the Dahl ss rat, we tested the function of α2-AR and NET by using UK 14,304 and Cocaine, respectively. Nevertheless, our data do not suggest dysfunction in these control mechanisms as a result of exposure to HFD over a period of time.

In our quest to find the underlying mechanisms for the development of HFD-associated hypertension in the Dahl ss rat, we also looked at the response to a direct nerve stimulation at varying frequencies. Although we found out that there was a transient increase (17-wk) in neurogenic constriction in females, that was not due to increase in neurotransmitter release, or due to augumented vascular reactivity. Similar study in obese and older male Sprague Dawley rat has shown a frequency-dependent increase in adrenergic and purinergic constriction (Haddock and Hill, 2011), which does not correlate to our finding from 17-wk female Dahl ss rat. Moreover, the transient

increase in neurogenic constriction contrasts to the lower purinergic response in females at the same time point. This remains to be explained since no one has done similar study in females.

To further evaluate whether HFD contributes to hypertension by altering the periarterial sympathetic nerves, we used immunohistochemical staining along with confocal imaging. Our data show that HFD does not change the adrenergic or purinergic nerve density in males and females. In addition, the lack of evidence from our HPLC data for NE content MA and plasma to show the effect of HFD or transient change suggest no link between periarterial nerves and increase in blood pressure due to HFD in the Dahl ss rat. Finding no clear indication for HFD-related hypertension from the prejunctional nerves, we moved on to look the postjunctional response.

Data from NE concentration-response curves from both sexes indicate lower vascular reactivity to NE at 24-wk regardless of diet. Similar result was found in ATP concentration-response curves from both sexes. This suggests that it is age but not necessarily HFD that reduces vascular reactivity in this rat model. One would expect that as these rats became more hypertensive with age, the vascular reactivity to decrease. However, that expectation assumes that the mesenteric bed contributes to the hypertension in this model. It is also possible that hypertension may decrease vascular reactivity with age.

The ability of MV to store a large volume of blood makes it an essential part of the circulatory homeodynamics. Like MA, MV is innervated by sympathetic nerves which control venous tone. Both mesenteric vessels function in concert to regulate blood pressure. In the case of MV, when it constricts as a result of increase in

sympathetic nerve activity, the blood stored in it channels back to the heart. This increase in venous return induces increase in cardiac output (CO), and redistributes blood to the resistance arteries with low compliance increasing pressure. High CO and TPR in turn lead to hypertension. In this light, we wanted to understand if the HFD-induced hypetension in the Dahl ss rats involves MV. In addition, we wanted to uncover the underlying mechanism for such changes. The primary focus was to investigate the sympathetic neurotransmission in the neuroeffector junction in MV.

Overall, the function of α2-AR is intact in MV similar to our finding from MA. Moreover, the function of NET is more or less not compromised as a result of HFD. These results suggest that the NE release and reuptake mechanisms are not affected by HFD or age in both sexes from MV. The absence of HFD influence on the neurogenic response except at 17-wk males (compared to CD) confirms at least the integrity of α2-AR. The difference between CD and HFD at 17-wk males is due to greater reduction in CD compared to 10-wk CD. Hence, we are not certain if the higher Emax in HFD versus CD at 17-wk males has a useful meaning.

The adrenergic and purinergic components were not affected at all by either HFD or age in both sexes. This may suggest that the neurogenic transmission from the prejunctional nerve terminal is not one of the reasons as to why the Dahl ss rat on HFD became hypertensive. Next, we investigated the venous reactivity to exogenous NE and ATP. Unlike in the MA, we observed sex difference in NE EC_{50} at 17- and 24-wk, i. e., EC_{50} was lower in HFD compared to CD at 17- and 24-wk in males suggesting greater vascular reactivity. In contrast, EC_{50} was higher in HFD versus CD at the same time points in females. There were some support for this sex difference from the TH-ir

immunohistochemical data. TH-ir nerve fiber density was not changed by HFD in males and females. Lower venous reactivity to exogenous ATP in HFD versus CD was clearly shown in females at all time-points. However, the pattern was not as clear in males. The NE content assessed by HPLC showed similar trend as in MA. Taken together, our data do not show a clear indication if the sympathetic nerves in MV are affected by HFD to the extent that blood pressure is increased.

There have been previous reports that shed some light into whether NE and ATP in the prejunctional nerve terminal are costored and coreleased from the same vesicles (and varicosities) or not (Sperlagh and Vizi, 1996; Stjarne, 2001; Burnstock, 2007; Kennedy, 2015; Sheng and Zhu, 2018; Mojard Kalkhoran et al., 2019). However, more work needs to be done to build a consensus around the localization of NE and ATP in the sympathetic nerve terminal. In addition, the population of sympathetic nerves projecting to the inferior MA and MV were identified to be localized in the center, and periphery of the inferior CG (Browning et al., 1999). Despite these findings, there has been a knowledge gap regarding the types of sympathetic nerve populations in the CG, and if the sympathetic distribution co-relates between CG and the mesenteric vasculature. To address these issues, we used immonohistochemistry and confocal imaging tools to investigate the symnpathetic nerves in the CG, MA and MV. More importantly, we tested the effect of HFD on the sympathetic distribution in CG, MA, and MV.

Our main findings include that: 1) there are three neurochemically unique populations of sympathetic vesicles and nerves in CG, MA and MV, namely TH-ir for NE, VNUT-ir for ATP, and TH/VNUT co-localization; 2) HFD does not alter the

abundance of these populations; and 3) there is no direct equivalent relation in the abundance of sympathetic neurotransmitters between CG and the mesenteric blood vessel. In conclusion, the presence of TH-ir has been known for some time; however in addition to that we showed VNUT-ir in the CG, MA, and to a lesser extent in MV.

Overall Conclusion: we have not found convincing and consistent evidence to support the hypotheis that the sympathetic neurotransmission is altered in the Dahl ss rat such that it is responsible for the HFD-induced hypertension observed in both sexes at from the hypertensive groups.

2.0 Significance

The current study was aimed at understanding the underlying relationship between hypertension and obesity. It is widely reported that increase in sympathetic neurotransmission in several organs such as the kidney, heart, and the brain is the driving factor in obesity-associated hypertension. The mesenteric circulation is part of the splanchnic circulation that is extensively innervated by peripheral sympathetic nerves. Moreover, significant amount of blood is trafficked through the mesenteric arteries and veins. Changes in arterial and venous tone due to chronic activation of sympathetic nerves affects the haemodynamics of blood pressure by decrreasing venous capacitance. Therefore, studying the underlying mechanism by which HFD is linked to hypertension in the mesenteric bed was an important effort. Furthermore, our experimental design was carefully planned to include both sexes, and three time-points in order to capture the development of HFD-associated hypertension in humans. Although we did not find a clear mechanism responsible for the HFD-associated hypertension in the Dahl ss rat, our findings provide us valuable lessons about the nature of this pathology and hopefully contribute a drop of knowledge to the field of hypertension research.

3.0 Research Limitations

Dahl ss rat is often used to study salt-related hypertension. It has also been used in stuides where by changes in salt and HFD levels were used to study hypertension and hypertension-associated metabolic disorders. In the current study, we used the same rat model and we exposed them to normal salt, but HFD. The rationale behind this approach was that by controlling the salt level, we can test the effect of HFD on the development of hypertension. We were cognizant of the fact that HFD-induced obesity can increase salt sensitivity due to damage to the kidney and alteration of the RAAS system. Therefore, the salt sensitivity of this rat model was in the picture when we designed our experiments. The focus of our research was to understand the underlying mechanisms for the development of hypertension in the mesenteric blood vessels. Specifically, we wanted to evaluate the sympathetic neurotransmission in MA and MV. Our model rats became obese with subsequent development of hypertension. This result was a success in itself; however, understanding the mechanisms for the HFD-associated hypertension required designing experiments to test our hypotheses that have demonstrated sex difference in sympathetic control of blood pressure at the systemic level, but no difference between diets or sexes was observed at the mesenteric level.

The main experimental designs included direct nerve stimulation using a pressure myograph system, immunohistochemical staining of sympathetic nerves, quantifying NE content in tissue and plasma by HPLC, and determining vascular reactivity to exogenous neurotransmitters. Our findings did not provide us with a consistent story to support our hypotheses. For example, the result from nerve density

count does not match our observation from the frequency response curves or from the vascular reactivity data. Despite stories from each data set, the inconsistencies from MA (CHAPTER 4) and MV (CHAPTER 5) made it challenging for interpretation.

The technical difficulty of the pressure myography presented a challenge at the beginning these studies; however, most of these data have been collected when the setup was properly mastered. It is worth mentioning that there have been equipment changes including the myograph chamber, and stimulator electrodes. Perhaps these changes made minor contribution to some of the data inconsistencies. Another concern is that data from some tissues were collected for multiple experiments at times for 6-8 hrs. Yet in some tissues fewer data were collected from them. The main issue here is that, the responsiveness or viability of the tissue may diminish with time. As a result, the data from these two groups may vary greatly affecting the overall assessment of the results.

Sharing animals have benefits in efficiently using several parts of the animal and reducing wastage. In addition, it allows for collecting data from different organs so as to understand the overall impact of the treatment on the health of the animal. In other words, it builds team work and cooperation which is an essential part of scientific research. The drawback is that it restricts doing some experiments, such as ovariectomy and nerve denervation if these and other procedures would interfere with experimental designs by other colleagues. However, this challenge can be overcome with continued dialogue with members who share the animals.

4.0 Future Directions

There are some experiments that can be done next that we believe will be helpful to better understand obesity-induced hypertension in the Dahl ss rat. First, ganglionic blockade with hexamethonium or ganglionectomy in 10- and 17-wk rats. This will allow us to evaluate the sympathetic control of blood pressure at these time-points and compare them to the 24-wk data our group has already published on (Fernandes et al., 2018). Moreover, we can compared males to females. Second, measuring food intake will provide us with additional information regarding the caloric intake. Third, assessing activity of the rats can help us evaluate energy expenditure. Fourth, measuring NE and ATP release amount via amperometry. Fifth, determining the contribution of β2-AR, localized in the VSMC, to the effect of HFD, age and sex on vasomodulation. Sixth, assessing endothelial function including the availability of NO. Seventh, looking into the effect of sex hormones by castration and ovariectomy. Finally, determining the effect of HFD, age, and sex on the function of sensory neurotransmitters (e.g., CGRP, and SP), and how this influences the net vascular response.

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