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A COMBINED HIGH SALT PLUS FRUCTOSE DIET MEDIATES HYPERTENSION

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A COMBINED HIGH SALT PLUS FRUCTOSE DIET MEDIATES HYPERTENSION

By

Taija Marie Hahka

A THESIS

Submitted in partial fulfillment of the requirements for the degree of

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In Biological Science

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Preface

Yuanyuan Fan has contributed to my thesis work by 1) assisting me with some pictures of the paraventricular nucleus and some brain sectioning work using the cryostat machine for my control group, fructose group, high salt group, and high salt plus fructose group.

Dr. Andrew Chapp has contributed to my thesis work by 1) obtaining cerebral spinal fluid from the rats, 2) prepping the samples, and 3) running ion chromatography for my results under the ion chromatography section for my control, fructose, high salt, and high salt plus fructose rat diet groups.

Note that none of this work has been published by a book or journal, but is intended to become published in the future.

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List of abbreviations

BBB blood brain barrier

BP blood pressure

CNS central nervous system

Dahl dahl salt sensitive rats

F fructose

GLUT5 glucose protein transporter 5 (fructose molecule transporter only)

HS sigh salt

HS+F high salt plus fructose

MAP mean arterial blood pressure

NS normal salt

OSM osmolarity

PVN paraventricular nucleus of the hypothalamus

qPCR or RT PCR Real Time PCR

SD Sprague Dawley Rats

SNA sympathetic nerve activity

SNS sympathetic nervous system

Abstract

The sympathetic nervous system (SNS) is the main control center for the neurogenic regulation of blood pressure and is affected in fructose induced hypertension. The brain is by far the greatest consuming and energy demanding organ in the body which has the ability to metabolize and generate fructose but with consequences. Diets high in salt and fructose enter the body and eventually crosses the blood brain barrier where it exerts its effects on SNS signaling. The aim of this thesis is to determine the connection between fructose and hypertension along with the detrimental effects of fructose within the brain. Here we test the hypothesis that a high salt combined with a high fructose diet contributes to hypertension by increased cerebral spinal fluid (CSF) sodium concentrations which alter key neuronal signaling mechanisms.

1 Chapter 1: Introduction

1.1 Overview and Significance

The increased intake of fructose in diets and the subsequent rise in hypertension are in a parallel escalation in today's western society[1]. It has been shown that fructose can provoke hypertension [1, 2], and that the central nervous system (CNS), particularly the sympathetic nervous system (SNS) exerts major regulatory control over blood pressure [3-5]. SNS dysfunction is also suggested as a component of fructose-induced hypertension. Fructose-induced hypertension is more prevalent in those who have had diabetes and obesity, and this can eventually lead to a more serious condition like cardiovascular diseases and an increased risk of mortality [6, 7]. According to the Center for Disease Control, cardiovascular disease killed nearly 801,000 people in 2017 and it's predicted to kill 836,546 people in 2018. Hypertension is the primary threat for cardiovascular disease, and in 2017 45.6% of US adults have hypertension per the American Heart Association[8].

It is well-established that elevated high salt plus fructose intake is the primary influence in essential hypertension and the onset of other metabolic syndrome disorders. It has also been established that fructose alone is responsible for alteration in brain genes [9, 10], synaptic plasticity, and can contribute to CNS impairment [11]. The majority of humans on the western diet with hypertension consume a larger quantity of salt and sugar. The more

salt and sugar one ingests the higher their blood pressure threshold will become. The less salt and sugar one consumes then the lower their blood pressure will be and this prevents risks for many future diseases. It is obvious that there are many factors when considering increased blood pressure. For example, added sugars increase the onset of obesity and bring about metabolic syndrome. Lanaspa 2018 et al. demonstrated that a high salt diet can activate the aldose reductase-fructose kinase pathway in both the liver and brain in mice. This study indicated that high salt can lead to endogenous fructose production and develop leptin resistance and hyperphagia, which is the origin for obesity, insulin resistance, and even fatty liver which are the hallmarks for metabolic syndrome. In addition, a combined high salt and fructose diet has been known to contribute to the onset of hypertension as well. Gordish K.L. and Beierwaltes W.H. 2017 have revealed that rats who consume a 20% fructose diet plus a high salt (4% NaCl) diet are predisposed to salt-sensitivity which leads to sodium retention, and increased blood pressure [12]. Studies of a high salt diet combined with a sugar diet (fructose) is much more realistic when comparing animal models to the current human’s diet habits. However, the molecular mechanisms that links salt and fructose to hypertension is poorly understood. The discovery of innovative mechanisms whereby a high salt diet may stimulate fructose uptake and may contribute to hypertension via neuronal apoptosis from the byproduct of acetate may clarify novel objectives for future treatments.

1.2 Blood Pressure Regulation, Hypertension and Mean Arterial Blood Pressure

According to the American Heart Association blood pressure by definition is “A pressure that the heart creates which allows the blood to move throughout the network of blood vessels including veins, arteries and capillaries[13].” Blood pressure variations can arise from many different modifiable factors including diet, smoking, alcohol, stress, exercise and more. However, it is possible that blood pressure can also be regulated by non-modifiable factors such as genetics, age, ethnicity, and family history. These blood pressure fluctuations, that our body experiences on a regular basis are monitored and regulated by our organism’s homeostatic mechanisms. The blood pressure homeostatic mechanisms are very well connected to each other. All mechanisms for blood pressure regulation include, but are not limited to: 1) the neuronal mechanisms of blood pressure regulation short term control, 2) the cardiovascular center via short-term control, and 3) the kidney’s hormone mechanisms for long term blood pressure control.

The CNS which contains the neuronal mechanism of short term blood pressure control has been demonstrated to be the primary control center for blood pressure regulation[5]. The CNS communicates with several organs in the circulatory system throughout the autonomic nervous system (ANS). The cardiovascular center can regulate blood pressure by managing cardiac output via regulating blood vessel diameter by vasodilation or vasoconstriction[5]. Although, vessel diameter can alter blood flow, blood

viscosity and the length of the blood vessel is also a major factor in determining blood flow resistance. The cardiac center may also regulate blood pressure by either stimulating sympathetic cardiac nerves to increase contractility or heart rate or inhibiting cardiac output by transmission through parasympathetic vagus nerves to decrease heart rate. Information about the state of the body can be received through baroreceptor and chemoreceptors by the cardiovascular center. Baroreceptors monitor arterial blood pressure and are primarily located in the carotid artery and sinus or in the aortic arch above the right atrium. Chemoreceptors are sensory neurons that monitor the body's oxygen and carbon dioxide levels located in the carotid bodies and aortic bodies[5].

1.3 Metabolic Syndrome and hypertension

Fructose-induced hypertension is more prevalent in those who have symptoms of metabolic syndrome [6, 7]. Therefore it is nearly impossible to discuss fructose-induced hypertension without mentioning metabolic syndrome. Metabolic syndrome is a cluster of cardiometabolic risk factors which escalates the risk for multiple chronic diseases including cardiovascular diseases, stroke, and type two diabetes [14]. The cluster of conditions that typically coincide with each other in metabolic syndrome include: hypertension, elevated blood sugar, excess body fat around the waist, obesity, abnormal cholesterol (ie. Low amount of high density lipids) and high triglyceride levels [14]. The metabolic syndrome has many etiological factors,

however, a central factor is a poor diet which consists of high amounts of fructose and elevated salt intake. A poor diet and sedentary lifestyle is the major attribution for the foundation of metabolic syndrome. High-carbohydrates and saturated fats may also be linked to metabolic syndrome. Therefore, there is no consensus of the direct cause of metabolic syndrome.

The popular way of describing the pathophysiology of metabolic syndrome is by discussing insulin resistance, which is the defect in insulin action which results in hyperinsulinaemia, which is needed to maintain normal glycemia. Insulin resistance has become an issue through the means of abundant fatty acids because the fatty acids released adipose tissue mass. Interestingly, we know that fructose is a lipogenic sugar that is directly metabolized in the liver, and either stays in the liver – contributing to a fatty liver—or transports directly to the blood stream [15, 16]. When a high concentration of fructose enters the blood it will directly be metabolized into fatty acids, triglycerides, lactate, and glycogen. This will travel around the body's vasculature until in either accumulates in the blood vessel walls and/or into the cerebral spinal fluid or makes its way into the brain via monocarboxylate shuttles resulting in an increased in brain lactate and acetate (data not yet published). When a high salt diet and high fructose diet is combined, the salt has the ability to stimulate the fructose uptake acquiring a salt-sensitivity, retaining sodium in the blood stream and creates an increase in blood pressure (demonstrated in rats) [12]. The high salt and fructose diet may be contributing to an insulin resistance and therefore leading to hypertension or vice versa, however, detailed

mechanisms are still unknown. Still, insulin resistance and fatty acids increase the production of glucose, triglycerides, and very low-density lipoproteins (VLDLs), which is very similar to how the body reacts to a high fructose corn syrup drink. Hypertension has been described in a variety of different ways with diverse proposed mechanisms. For example, since insulin is a vasodilator, and has the ability to create sodium reabsorption in the kidney, the vasodilator effects of insulin may become lost, and sodium reabsorption is conserved [15, 16]. Fatty acids have the ability to mediate vasoconstriction, which might be why vessels are damaged, and the side effect is an increase in blood pressure. Also, hyperinsulinemia may be related to an increase of sympathetic nervous system activity and ultimately contributing to hypertension [15, 16].

Metabolic syndrome and its components are very intricate; however, abdominal obesity appears to be an important contributing factor. In some theories, it has been presumed that visceral adipose tissue creates a flux of adipose tissue-derived fatty acids transferred to the liver from the splanchnic circulation, which is expected [15, 16]. This tends to increase the subcutaneous fat, releasing lipolysis into the systemic circulation instead of utilizing direct hepatic metabolism, similar to when we eat a high fructose diet. Although fructose is metabolized directly in the liver, the abundance is so shocking to the liver that it pours into the blood stream resulting in an increase in fatty acids contributing to visceral fats [15, 16]. Remarkably, the

4% NaCl plus 20% fructose drinking water has been show to generate obesity in Sprague Dawley rats in just three weeks (data not yet published).

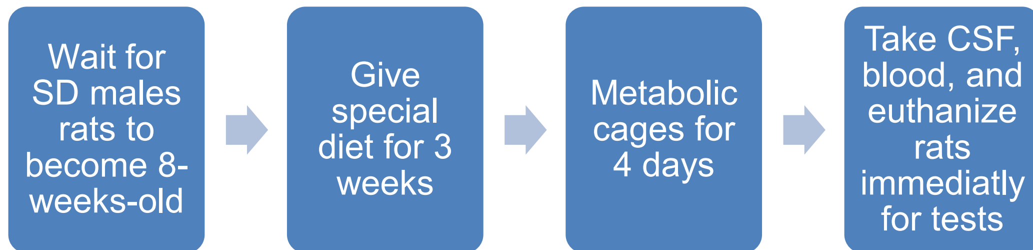
1.4 The relationship between rats and humans and their diet intake

The relationship between rats and humans can be contemplative in science. Often times the rat model compared to the human is not explained very well. In this thesis, this thesis will iterate why the comparisons between a rat and human are performed.

The rat model is the preferred model of human cardiovascular and neurosciences[17]. This is because the physiology of the rat is simple to monitor, along with the fact that we have accumulated an enormous amount of data[17]. It has been demonstrated that the rat model regarding physiology is close to the human for examples, cognition is superior in the rat and the rat's neuro circuitry regarding memory and learning is quite comparable to the human[17].

Interestingly a rat's age can also be compared to the human age[18]. Per Robert Quinn one human year is comparable to about 10 rat days for adult rats which they're considered to be adults when they reach sexual maturity around 6 weeks[19]. In this thesis we have used 8-week-old rats which are 2 weeks past sexual maturity/adult hood but this is comparable to a ~18 year old human[19] when you use the calculations that during the rat adult phase

11.8 rat days equals ~1 human year. The following flow chart below demonstrates the outlined ages of the rats for this thesis.



In this thesis we have used 1 control diet and 3 special diets. The normal salt (NS) or control group was fed 0.4% NaCl chow and normal drinking. The high salt (HS) group was given a 4% NaCl diet with normal drinking water. The fructose (F) group was given a normal salt chow with 20% fructose in their drinking water.   Finally, the high salt plus fructose (HS+F) rat group was given a 4% NaCl chow plus 20% fructose in their drinking water. The water was from the tap and then was autoclaved. The fructose was added after the water was autoclaved. The fructose water bottles were changed every three days to prevent any bacterial growth. The diagram below indicates the approximate value of water and food intake per each day for each group (from my metabolic data, this is the averaged data for each day of consumption, whole numbers will be used for simple mathematics). This will

be further used to explain why how diet is comparable to humans. Data for metabolic cages are in the results section of this thesis.

The approximate value of food intake for SD adult male rats after a three weeks of a special diet treatment data from metabolic cage measurements (rounded to the nearest whole number)								
Rat Group	**Normal Chow**	**4% NaCl Chow**	**20% fructose water**	**Normal water**	**Calories from chow**	**Calories from fructose water**	**Total calorie intake**	**Average body weight of rat**
Control (NS)	22 g	X	X	25 mL	70 Kcal	X	70 Kcal	377 g
High Salt (HS)	X	23 g	X	45 mL	71 Kcal	X	71 Kcal	376 g
Fructose (F)	15 g	X	19 mL	X	48 Kcal	76 Kcal	124 kCal	364 g
High Salt and Fructose (HS+F)	X	13 g	34 mL	X	40 Kcal	136 Kcal	176 kCal	355 g

Based off the following custom diet from Envigo:

- *4.0% NaCl= 3.1 Kcal/g Envigo TD.92034*
- *0.4% NaCl=3.2 Kcal/g Envigo TD.96208*
- *1 gram of fructose is equal to 4 Kcal/g*

In the above table the relationship between kilocalorie (Kcal) and fructose consumption was made. Body weights were given for reference. Sprague

Dawley (SD) rats are comparable to healthy humans without any diseases or health issues. For the control diet (NS, normal salt diet, 0.04%), this would be relative to a healthy human diet. Including vegetables, protein, minimal carbohydrates, and no processed sugars while only drinking water. The high salt (HS) diet is comparable to a human diet who consumes 4% salt or the equivalence of 120,000 mg of NaCl with normal drinking water per day, no sugary drinks. The 20% fructose diet (F) would be comparative to a human diet who drank 500 calories of fructose sugary drink, whose total calorie consumption is based on a total of 2500 calories per day. Finally, a high salt and fructose diet would consist of a human to eat a combination of about 120,000 mg of NaCl plus drink 500 calories of fructose per day based on a calorie diet of 2500 calories/day.

2 Chapter 2: Fructose Effects on the Central Nervous System; Review Article

2.1 Abstract

Fructose has detrimental effects on human physiology, and has been linked to obesity, cardiovascular diseases, and hypertension. However, the link between fructose-induced hypertension within the central nervous system (CNS) is poorly understood. The objective of this paper is to shed light on the research linking the contribution of fructose to CNS control of blood pressure. This paper aims to elucidate the connection of fructose to inflammation, and fructose leads to the elevation of lactate in the CNS which ultimately contributes to hypertension. A comprehensive overview of metabolism of fructose within the body and CNS are discussed. The by-product of reactive oxygen species (ROS) and nitric oxide (NO) from fructose feeding within areas of the brain are discussed as well. This paper discusses the issue that fructose has devastating effects on astroglial, neuronal, and microglial cells. Furthermore, the molecular mechanisms of fructose induced inflammation that regulate histone deacetylases3 (HDAC3) - toll-like receptor 4/ nuclear factor-kB (TLR4/NF-KB) pathway is discussed as one of the main molecular pathways in which hypertension is induced in the paraventricular nucleus (PVN). Finally, this paper brings to light that fructose and salt are synergistically interconnected in the intestine and kidneys shown by glucose transporters and sodium hydrogen exchanger (NEH3) expression.

2.2 Introduction

The limitless escalation of fructose in today's western world diet is worrisome because it is related with a parallel increase of obesity[20, 21], hypertension[22], and cardiovascular diseases[23]. Fructose is incorporated into our foods by a more common additive known as high fructose corn syrup (HFCS), which is a high level processed sugar including at least one genetically modified organism (GMO)[21]. Specifically, fructose is a glucose sweetener used as an alternative for sucrose and can be found in pops, condiments, juices, baked goods, and candies[24]. Although a lot of the population has started to recognize that HFCS is linked to weight gain, hypertension, and cardiovascular disease some brand name products have renamed "high fructose corn syrup" on their food labels to "natural sugars" to trick buyers into consuming their products and making unhealthy decisions[25].

Fructose is a ketonic monosaccharide consisting of a five-member ring structure with the same molecular formula of glucose ($C_6H_{12}O_6$), which instead is a five-member ring structure and an aldehyde[24]. Fructose is commonly derived from sugar beets or sugar canes and is present in small amount of fruits and honey[24]. It is well known where we can get fructose or HFCS, but the detailed metabolism and transportation of fructose around the body seems to be a greater mystery.

Fructose is transported passively via facilitated diffusion by a glucose transporter called GLUT5 [26-28]. GLUT5, a uniporter, is special in the way that

is can solitarily transport fructose and does not have the capability to transport glucose or galactose.[26, 28, 29]. GLUT5 is highly expressed in the small intestine while there are minimal quantities of GLUT5 expressed in the brain, adipose tissues, sperm, skeletal muscles, and kidneys. Interestingly, the chronic ingestion of the GLUT5 substrate fructose has been linked to disorders like type two diabetes, hypertension, non-alcoholic fatty liver disease (NAFLD), inflammation, and obesity[28, 30].

GLUT5 is only capable of transporting fructose but another glucose transporter, GLUT2, is the only other glucose transporter which can also transfer fructose but with a caveat [31]. The caveat is that GLUT2 does not solely transport fructose, it has the ability to convey glucose and galactose as well [31]. GLUT2 is also a facilitative glucose transporter and has been discovered to be located in the pancreatic beta-cells, liver, intestinal mucosa, kidneys, and in the hypothalamus area [31, 32]. It seems that between GLUT2 and GLUT5 expression, GLUT5 may have a larger connection to inducing hypertension rather than any other GLUT.

Many clinical studies have been leaning toward the role of the CNS as one of the main connections contributing to the onset of fructose-induced hypertension [3, 33] However, the connotation among salt stimulated fructose induced-hypertension is still lacking in definition because current studies have contributed very little to these findings. A common fructose-induced hypertension study was done by Farah V. and researchers who have demonstrated that a fructose diet

increases mean arterial pressure (MAP) and disrupts insulin homeostasis of male Wistar rats when fed a 66% fructose diet over ten weeks [34]. To shed light on salt stimulated fructose-induced hypertension, Gordish et al. 2017, demonstrated that systolic blood pressure was increased when salt (4% NaCl chow) was added to the 20% fructose water diet in Sprague Dawley (SD) rats compared to the control, fructose alone, and salt alone [35].

This review will aim to focus on the role of fructose and its effects on the central nervous system, by evidence of several fructose feeding animal studies. Since fructose is transported by GLUT5, the expression of GLUT5 in the brain and spinal cord will be assessed. The effects and actions of fructose on the CNS lead to increase in food intake will also be discussed. A comprehensive evaluation of fructose metabolism in the body and brain will also be discussed.

2.3 The pathway of fructose metabolism

Fructose-1-phosphate pathway, and fructose-6-pathway are two of the passageways that fructose is metabolized. Fructose 1-phosphate pathway requires ketohexokinase/fructokinase, an enzyme that catalyzes the transfer of a phosphate group from ATP to fructose. The results of fructose 1-pathway by the enzyme fructosekinase traps fructose's metabolism in the liver. The fructose 6-phosphate pathway is an intermediate of glycolysis and gluconeogenesis. However, the fructose-6-phosphate pathway has a low affinity for fructose and a high Km value which is unfavorable. Km is the concentration of substrate in

which the enzyme is able to reach half of the catalyst rate constant[36]. The preferred pathway for fructose metabolism is the fructose-1-phophate pathway which is located almost entirely in the liver.

Fructose is almost completely metabolized in the liver because of the favored fructose-1-phosphate pathway due to the low Km value and high affinity for fructose [26, 37]. One of the resulting products from fructose metabolism in the liver is glycogen[38]. Liver glycogen is considered to be a fuel reserve and can easily be broken down into glucose molecules when needed[38]. However, HFCS can often cause the liver to be overwhelmed and that can create a huge burden resulting in hepatic steatosis (fatty liver). When the liver is stunned with excessive fructose then that fructose will become converted into free fatty acids via *de novo* lipogenesis and stored as endogenous triglycerides or very low-density lipoproteins (VLDL)[26, 37, 39]. The storage of fatty acids, VLDL and triglyceride are eventually stored as fat within the liver[39]. The metabolism burden of fructose on the liver results in the increase of endoplasmic reticulum (ER) stress and reactive oxidative species (ROS) production, known to stimulate insulin resistance[39]. Fructose may also be metabolized in the intestine[29], kidney, skeletal muscles, testes[29], and the brain.[39]. The metabolism of fructose in the brain is further discussed in the next section.

2.4 The metabolism of fructose in the brain

Fructose is not only metabolized in the liver or intestine, it is also metabolized by other tissues including the brain which has been shown in oxidative assays which measures oxidative stress of dissected brain regions from adult mice [12, 18]. Sarah A. Oppelt et. al. and Funari V.A. et. al. discovered that the cerebellum, hippocampus, cortex, and olfactory bulb of adult mouse brain slices included a substantial number of neurons and glial cells comprised of expressing genes for fructose-1-phosphate pathway cascades [12, 18, 20]. This suggests that fructose may be metabolized in those brain areas [12, 18, 20]. Even in the hypothalamus, the brains metabolic control center, has been recognized in fructose metabolism which contributes to alterations in gene activity [17, 18].

The human body and brain both contain aldose reductases, and sorbitol dehydrogenases which has the ability to reduce glucose to sorbitol and oxidize sorbitol to fructose, respectively [40]. Sorbitol ($C_6H_{14}O_6$) is a type of sugar alcohol which was extracted from glucose and is commonly used as an alternative sugar in foods[41]. This ultimately means that glucose can be converted to sorbitol and then further oxidized to fructose. This is considered to be the polyol pathway, an alternative glucose pathway [40].

For some reason the brain can bypass the rate-limiting step in glycolysis but with consequences. This rate-limiting step results in the phosphorylation of fructose-6-phosphate to form fructose-1,6-bisphosphate. The purpose is to

convert fructose directly to malonyl-CoA, which requires energy to elongate the fatty acid chain[42]. The result is that the metabolism of fructose in the CNS rapidly depletes hypothalamic adenosine triphosphate (ATP)[42]. The exhaustion of ATP in the hypothalamus will then lead to the upsurge of adenosine monophosphate (AMP) and initiation of adenosine monophosphate kinase (AMPK) signaling, plus increase of food intake which can result in the consequence of weight gain[42]. Interestingly, it is unclear whether fructose crosses the blood-brain barrier but it’s known that fructose can be endogenously produced from glucose to sorbitol and finally to fructose [19]. However, according to researchers Janice J. Hwang et. al. from Yale University the polyol pathway subsidizes fructose production in the CNS from glucose, which is related to intracerebral fructose changes and is not correlated to the human plasma fructose levels[43].

2.5 Fructose effects on the CNS

Overwhelming publications demonstrate that the CNS play a more imperative role in the control of blood pressure and cardiovascular function than was previously thought. The CNS is responsible for regulating the sympathetic nerve activity (SNA) and blood pressure which directly contributes to dissimilar types of hypertension [44]. In some way the CNS indirectly contributes to all categories of hypertension but the mechanisms have remained undistinguishable. Remarkably, the CNS is considered to be the main regulator

for blood pressure control due to the sympathetic nervous system (SNS) which is part of a larger system called the autonomic nervous system (ANS).

2.6 Fructose effects on obesity related hypertension

Melanocortin, a collection of peptide hormones derived from the pro-opiomelanocortin (POMC) in the pituitary gland, and its receptor activities in the brain has been demonstrated to regulate blood pressure and the SNS activity in humans and animal models [33, 45, 46]. POMC neurons tend to result in a decrease of food consumption along with weight loss[47]. However, the activation of neuropeptide Y and agouti-regulation protein (NPY-AGRP) neurons consequence in weight gain and increased food consumption[47]. Melanocortin-4 receptor (MC4R) has been known to play a role in body weight regulation, feeding behavior, the regulation of metabolism, and blood pressure regulation [46]. MC4R is the key mediator of the two neurons POMC and NPY-AGRP and has an effect to determine which will be activated specifically in areas including, but not limited to, the paraventricular nucleus (PVN)[47].

Obesity-related hypertension is highly linked to MC4R and has shown to be MC4R dependent[48]. Animals with hypertension and an increased SNS activity were given a blockade of MC4R which decreased blood pressure, heart rate, and the SNS response. MC4R is important for the control of food because it mediates NPY-AGRP and POMC neurons which is linked to the initiation of signaling cascades involved with leptin receptors. Leptin is primarily involved in

the regulation of food consumption. It is a hormone secreted from white adipose cells that is associated with inhibiting the hunger sensation by acting on receptors in the arcuate nucleus of the hypothalamus (ANH). Leptin intravenous infusions have been found to increase blood pressure in rodents with severe obesity[49]. The leptin obese rodents produced continuing increases in blood pressure (6 mm Hg after 7 days), heart rate, and adrenergic activity[50]. Clinical studies on hypertensive humans have shown that elevated plasma leptin has a correlation between high levels of leptin which occurred in hypertensive patients compared to non-obese normotensive patients [49, 51]. The CNS links the MC4R-expressing neurons to the POMC and NPY-AGRP neurons by projections in the area of the PVN. MC4R is located on the axon terminal of the PVN and can increase the amount of gamma-amino butyric acid (GABA) that can be released to the neurons of the PVN, and has a strong connection to obesity-related hypertension [48]. When GABA, a neurotransmitter, is secreted from NPY/AGRP neuronal axon terminals this will block the POMC neurons ultimately resulting in weight gain and uncontrollable feeding[52]. The increasing levels of leptin in obese hypertensive humans are in correlation to increased MCR4 in the CNS [53, 54]. Obesity is linked with increased levels of MC4R which is known to be related to increased activity of renal SNS and increased sodium absorption which further associates it to induced hypertension[54].

2.7 Fructose effects on the CNS contribute to hypertension

Fructose is vastly connected to obesity and hypertension in the today's western society [20, 55]. NPY itself is involved in enhanced food intake, involved with obesity, and is the most abundant peptide in the CNS[53]. MC4R-expressing neurons are involved in both hypertensive and obese rodents and humans[54]. Fructose is consistently used in our daily processed foods and although fructose is mainly metabolized in the liver, small amounts are metabolized in the intestines and will contribute to enhance feeding due to continuing MC4R expression of neuron in the NPY. Taken together these all suggest that CNS plays a more critical role in the control of fructose-induced hypertension [33, 45, 46].

The CNS play an imperative role on fructose-induced hypertension by the involvement of neuronal nitric oxide synthase (nNOS) in the NTS, reactive oxygen species (ROS) in the RVLM, and downregulating superoxide dismutase SOD1 and SOD2.

2.8 The role of a high fructose diet on nitric oxide production

Nitric oxide is considered to be a radical gas, but it has an important role in biological processes such that it is an important molecule in signaling cascades. [56, 57]. There are three types of nitric oxide synthase which are neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS)[56].

NO in the blood vessel wall is produced via the cascade of the calcium-calmodulin scheme; endothelial nitric oxide synthase (eNOS) is critical for preventing damage to the walls of vasculature[58, 59] Particularly, eNOS dysfunctions have been linked to hypertension as well [58]. However, there are other areas in your body such as in the CNS where nitric oxide plays a critical role in synaptic plasticity, sleep-wake cycles, and the regulation of hormone cycles [56, 58].

Within the CNS of a healthy individual, nNOS, reacts with any superoxides (O_2•-) that have accumulated from risk factors like fructose-induced hypertension or glucose increases[56]. Following this reaction, nNOS and O_2•- will then combine together to form peroxynitrite, which reduces ROS scavenging capabilities for the neuron cells [58]. When the body undergoes salt consumption or obesity, sympathoexcitation occurs from oxidative stress in the rostral ventrolateral medulla (RVLM). Fructose has been shown to stimulate sympathetic over activity and excite the brain during fructose intake. However, when adult normotensive rats were subjected to an 8 week diet of fructose (increased risk factor), nNOS developed a vicious cycle and eventually generated enzymes that produced superoxides[60]. The tissue of the rostral ventrolateral medulla (RVLM) level of NO was shown to decrease and ROS production increased simultaneously [60]. nNOS protein expression in the RVLM was significantly increased after the 8 week diet of fructose intake of these rats[60]. The superoxides that started to build up in the RVLM eventually

resulted in free radicals and the consequence that of oxidative stress, leading to fructose-induced hypertension and sympathoexcitation [60].

nNOS has been linked to hypertension due to that fact that nNOS is dominantly contributed to the control of intracellular calcium homeostasis in the heart[57] and in the CNS[61, 62]. Blood pressure is highly regulated by intracellular calcium because an influx of calcium into vascular smooth muscles can result in an intensified muscular morphology, tone, and increasing vascular resistance thus resulting in elevated blood pressure[63, 64]. Cirello J et. al, 2012 has revealed that a calcium regulating glycoprotein hormone stanniocalcin-1 has been demonstrated in the nucleus solitary tract (NTS) was involved in the regulation of neurons that participate in controlling arterial blood pressure via the baroreceptor reflex[65]. It has been demonstrated that extracellular-signal-regulated kinase (ERK) is involved in the regulation of blood pressure in the NTS[62]. With this evidence, it is indicated that the CNS plays an important role in hypertension from the contribution of nNOS and eNOS synthases.

Other studies have showed that microinjection of angiotensin I and angiotensin II into the third cerebral ventricle of the brain considerably elevated blood pressure compared to the administration of the drugs in the peripheral blood of rats[66] and dogs[67]. These results further demonstrate that the elevation of blood pressure originated from activating neuronal mechanisms rather than peripheral vasoconstriction activities[66]. Specifically, it has been demonstrated that brain angiotensin II is one of the notable contributors which

links the CNS to reactive oxygen species-dependent hypertension (discussed later) [67].

2.9 The role of a high fructose diet and ROS production

Interestingly, ROS is generated from neuron cells itself and is also generated by microglial cells and astrocytes which can transform communication to and from neurons by inducing long-term potentiation or modulating synaptic plasticity [68]. We have mentioned above that GLUT5 is localized in microglial cells and it's capable of depleting ATP levels and increasing AMP levels in the CNS [69]. However, during the depletion of ATP levels, fructose is eventually metabolized to uric acid and uric acid has been found to stimulate ROS by the activation transforming growth factor beta-1 and nicotinamide adenine dinucleotide phosphate (NADPH)[70]. Fructose has also been linked to intracellular phosphate depletion as well as a significant decrease in AMPK phosphorylation. A rise in intracellular uric acid is proficient in producing proinflammatory effects on the CNS, liver, and vascular cells. For example, metabolizing fructose during a fructose load generates a surge in intracellular uric acid levels, and with a continuous consumption of fructose intake signals your body to produce fructose enzymes. The fructose enzymes allow your cells to become sensitive which contributes to an overproduction of uric acid known as hyperuricemia, triglycerides and free radicles[71]. Fructose, which produces uric acid as a byproduct when metabolized, has been linked to metabolic diseases particularly in connection with hypertension. The amplification in uric acid has

been shown to decrease nitric oxide (NO) levels and is likely the incentive for oxidative stress[71].

Keep in mind that the microglial cells are a specialized type of macrophages within the CNS and contributes to an enormous part of the inflammatory and immune responses of the CNS during fructose loading [41]. High levels of fructose contributes to elevated levels of intracellular uric acid which can induce oxidative stress in the CNS, vascular cells and liver. When oxidative stress is induced, the macrophages in the CNS produce a response via signaling molecules. However, when reactive oxygen species (ROS) occurs during fructose loading the cell's oxidative damage results in intracellular DNA damage, this sends out a stress response to release the pro-inflammatory cytokines (PIC)[72]. The increase in oxidative stress and ROS are both important pathogenic influences regarding hypertension development and diagnosis [56]. ROS that is produced in the neuronal, vascular system, or renal system is able to influence signaling molecules and can lead to hypertension [56]. For example, when rats were fed 10% fructose for 4 weeks they formed fructose induced hypertension and their ROS levels in the nucleus tractus solitarii (NTS) tissue were significantly greater and the NO levels were significantly reduced [2]. When ROS occurs during fructose loading the cell's oxidative damage results in intracellular DNA damage which sends out a stress response to release pro-inflammatory cytokins (PICs)[72].

2.10 The role of high fructose and antioxidant imbalance

When free radicals are formed in the body antioxidants can inactivate/quench them. Superoxide dismutase 2 (SOD2) is an antioxidant enzyme within the mitochondrial matrix that has the ability to destroy superoxide anion radicals that are toxic to the neurons [2]. The mutation of *SOD2* has even been linked to neurodegenerative diseases like amyotrophic lateral sclerosis or leigh syndrome[73]. SOD is usually the immediate defense against ROS production and the decrease of *SOD2* indicates that fructose feeding promotes ROS imbalance [2]. *SOD2* was demonstrated to be significantly down regulated in the NTS of the fructose fed (10%) rats compared to the control while their systolic blood pressure become elevated (~15mm Hg compared to control)[2]. Fructose feeding for 4 weeks in rats enhanced ROS production in the NTS and that has been linked to neuropathogenesis of hypertension, neurodegenerative diseases, and the pathophysiology or cardiovascular diseases [2]. There are other sources of ROS that maybe linked to ROS-dependent hypertension; for example NADPH oxidase (NOX) [66, 67].

2.11 Fructose and the blood brain barrier

In the CNS, cells are responsible for their own energy, metabolizing glucose and lipids. However, the brain alone is one of the most energy demanding organs in the human body because it is full of neurons that starve for glucose. It is well known that glucose is the primary substrate needed for the brain to carry out its functions and that glucose can cross the blood brain barrier

by the help of glucose transporters. Although there seems to be a large debate in regard to fructose - fructose can pass through blood brain barrier? This section does not choose one side instead you will see the data behind both ideas.

8 healthy women and men were given a 20% dextrose (sucrose and fructose mixture) though a constant 4 hour intravenous injection to their arm, the dextrose levels were held at the same concentration (220 mg/dl) for 4 hours long [20]. Janice J. Hwang et. al. found that these participants had high levels of intracerebral fructose levels by magnetic resonance spectroscopy (MRS) scanning[43]. Furthermore, they discussed that the reason is due to the polyol pathway which suggests that fructose is produced endogenously in the human brain from the process of glucose to sorbitol to fructose [43]. In another study from Janice, the fructose measurements within the cerebral spinal fluid were 20 times higher compared to the concentration of plasma fructose levels from healthy and obese pregnant woman [40] suggesting that the polyol pathway is involved in fructose endogenous CNS creation. In both studies Janice J. Hwang et. al. suggested that fructose is generated from glucose in the human brain during hyperglycemia[40][20]. Results from Janice J. Hwang et. al. experiment indicated that glucose levels mediate fructose levels in the brain. In addition, any increase in blood glucose contributed to fructose's effects on the brain [20] . This compelling evidence demonstrates that fructose may not pass through the blood brain barrier rather fructose can be generated by the brain.

Contradicting experiments indicate that fructose can enter the brain by a presence of GLUT5 in the blood brain barrier in rats that consume a high fructose diet [26]. Shu HJ et. al. has shown that brain cerebrum mRNA levels of GLUT5 increase 1.5-fold in male rats after only seven days of fructose feeding[26]. It is suggested that fructose can be utilized as an alternative energy source in the brain during a time of critical need [22, 26, 27]. With the provided data, fructose was demonstrated to cross the blood brain barrier when fructose was given though ingested (feeding) and given by an intraperitoneal injection [42, 74].

2.12 Fructose Causes Lactate Elevation Which May Contribute to Hypertension

When 10 volunteers received a 10% intravenous (IV) dose of fructose at 0.5 grams per kilogram of body weight per hour for two hours it was found that their serum lactate rose significantly during this fructose administration[75]. Interestingly, lactate is able to cross the blood brain barrier (BBB), and can easily be taken up by the brain[76]. When lactate levels rise in the blood from IV fructose[76], then we can assume that there is a net influx of lactate from the blood into the brain. Elevated blood lactate levels have been linked to hypertension especially in women[77]. In another study, blood lactate was elevated twice as much in obese-hypertensive patients compared to their lactate levels after weight loss[78]. It seems that lactate levels fluctuate with blood pressure, when patients are hypertensive they had elevated lactate levels in their blood[78]. Fructose given by IV was shown to increase blood lactate, with what

we know lactate can cross the blood brain barrier[76], and can definitely influence hypertension. Lactate is a product of anaerobic metabolism and is a link between oxidative metabolism and glycolysis and furthermore after lactate is formed, pyruvate can be converted to glucose in the gluconeogenesis pathway[79]. It is known that muscles can generate lactate especially during high intensity exercising, and also that the heart, liver and kidneys can use lactate as fuel[79]. However, in the brain the role of lactate in sparing amounts may be used in assistance to neuronal action potentials firing rates. When lactate is in the brain in large quantities it may be linked to neurodegenerative diseases and is not used as an energy source[79]. In the brain, lactates utilize an astrocyte-neuron lactate shuttle which has been hypothesized that "astrocytes metabolize glycogen and glucose to lactate which is transferred to neurons through the axons juxta synaptic process according to Smith et al. 2003. [79]" Since hypertension is regulated by mechanisms in the SNS[78] which are ultimately controlled by the CNS we can conclude that fructose[75] causes lactate elevation this lactate then leads to the CNS transferring across the BBB [76] into neuronal cells creating excitation and neuronal apoptosis contributing to the possible onset of hypertension.

2.13 Fructose in the CNS is linked to inflammation

GLUT5 has been shown to be expressed in the CNS, and it has the ability to transport fructose in microglial cells. The transportation of GLUT5 may have an effect on glial and neuronal interactions. For example, glial cells can multiply

when the brain experiences inflammation, in a sense this also occurs when angiotensin II brings about hypertension [21] and fructose induced hypertension. A high fructose diet has been linked to inducing hypothalamic proinflammatory cytokine (PICs) production and astrogliosis, which is the increase in astrocyte production due to the fate of neighboring neurons becoming traumatized, infected, or inflamed[22]. According to Jian-Mei Li et. al., fructose induced astrocytosis inflammation was associated with the TLR4/NF-kB pathway, which is the main control of hypothalamic inflammatory response and controls the expression of cytokine genes [80]. Jian-Mei Li et. al. further suggests that increased histone deacetylase 3 (*HDAC3*) is associated with fructose induced inflammation by activation of NF-kB transcription regulator and that the suppression of *HDAC3* overexpression restored fructose neuronal injury[81]. In addition, the suppression of TLR4/NF-kB pathway also showed restoration from fructose induced neuronal injury in brain astrocytes [80]. Remarkably, this demonstrated that theTLR4/NF-kB pathway and HDAC3 are interconnected due to hypothalamic fructose induced neuronal inflammation [80]. Fascinatingly, NF-kB has a primary role in hypertension because NF-KB increased the expression of pro-inflammatory cytokine (PICs) gene creating inflammation in the PVN which contributes to hypertension [80]. The connection that inflammation in the brain occurs and the result is the onset of hypertension is due to a large variety of rat models, which confirms that abnormal inflammation in the brain contributes to the rise in mean arterial blood pressure and that respond to the inflamed vasculature in the brain [25-27]. When rats were intracerebroventricularly (ICV) infused with

angiotensin II, the brain renin-angiotensin system activated NF-kB in the PVN which then contributed to an increase in oxidative stress, mean arterial blood pressure, PICs, aldosterone and norepinephrine [28, 29]. What is unique is that *HDAC3* has the ability to bind to NF-kB inhibitory subunit α which increases transcription, this resulting in inflammation and then permitted the macrophages to yield an overwhelming supply of cytokines in the PVN [80, 81].

Brain astrocytes and microglial express GLUT5, the main glucose transport protein for fructose, and are highly localized in the brain[30]. Astrocytes in the PVN and superaoptic nucleus (SON) display great anatomical plasticity and are capable of extensive transformation [82]. Astrocytes also play an important role in synaptic plasticity and metaplasticity mechanisms [83]. Nevertheless, GLUT5 expression has been particularly linked to synaptic activity dysfunction due to fructose induced hypertension and high fat plus high fructose diets [83]. During the 4% high salt chow plus 20% fructose drinking water diet in SD rats for 3 weeks, GLUT5 expression in the PVN was increased dramatically compared to control, 4% high salt diet, and 20% fructose alone diet (data not yet published). When rats were fed a high fat and high fructose diet for 8 months there was a noticeable reduction in spine density, synaptic plasticity, and low levels of brain-derived neurotrophic factor (BDNF) [84]. In another study, adult male SD rats were given a high fructose solution alone and that showed an impact on synaptic plasticity by utilizing a synaptic growth marker, synaptophysin, which was measured and found to be decreased during a high

fructose diet. According to Shi et. al., when fructose induced hypertension occurred there was an activation of glial cells and an increase in proinflammatory cytokines in the paraventricular nucleus (PVN) of the brain [85]. The inflammation from hypertension recruits the glial cells to develop properties similar to that of macrophages [86]. This includes glial cells which then release chemical mediators that may be associated between neuron cells and synaptic strength, density, and size [86]. As stated by Pedro Cisternas et. al., an observed decrease in postsynaptic density (PSD) from 68 nm to 33 nm was indicated in fructose-fed groups of mice as well [11].

2.14 Model: fructose and high salt mediates hypertension

It is believed that fructose and salt synergistically have a hypertensive effects[87]. Fructose stimulates salt absorption in the small intestine specifically the jejunum, and the kidney [88]. Interestingly, it is thought that GLUT5 is responsible for enhancing the absorption of fructose and salt in the intestine [27, 87]. Enterocytes lining the villus of the intestine have used GLUT5 as the major fructose transporter to pass fructose, rapidly, through the apical membrane [26]. GLUT5 wild type mice when given a high fructose diet (60% fructose) for a total of 14 weeks have increased salt absorption is facilitated through increased expression of a putative anion transporter 1 (PAT1) and sodium hydrogen exchanger (NHE3) in their jejuna and kidney tubules. [87]. In addition, Sharon Barone et. al. then demonstrated that the GLUT5 wild type mice with a high salt and high fructose diet have developed systemic hypertension compared to the

GLUT5 knockout mice [27]. Fascinatingly, there is no known research that aims to find whether the brain synergistically uses fructose and salt combination to bring about the onset of hypertension. Given this, we live in a day where our diets include high fructose corn syrup (HFCS) and substantial amounts of salt, but the molecular effects of the high salt and fructose in the CNS remain in unclear.

2.15 Review Article Conclusion

These reviewed studies and metabolism of fructose in the body and brain suggest that fructose plays a large role on the effects of the central nervous system. We also suggest that fructose effects the CNS, not only by its ability to generate fructose from glucose, but also that high fructose levels can disrupt glial and neuronal interactions resulting in consequences. Our future study will investigate whether or not fructose and salt synergistically mediate hypertension. We will focus on fructose effects of the CNS and ultimately aim to find fructose's contribution to hypertension.

3 Chapter 3: Methods

3.1 Animal Models, Diets, Blood Pressure, and Metabolic Cages

Male rats were purchased from Charles River Laboratories in Wilminton, MA. Sprague Dawley (SD) eight-week-old rats were started on a specialized diet including 1) normal chow (0.4% NaCl, Envigo RMS, IN, USA) with normal drinking water, 2) high salt chow (4% NaCl, Envigo, RMS, IN, USA) with normal drinking water, 3) normal chow with 20% D-(-)-fructose (Sigma-Aldrich) drinking water, and 4) high salt chow with 20% D-(-)-fructose (Sigma-Aldrich) for a total of 3 weeks. The tap water was autoclaved for 15 minutes at 150°C prior to adding the fructose into it and then stored in a 4°C fridge. Fructose was not autoclaved in this experiment. Fructose water bottles were changed every 3 days to prevent mold from growing. Prior to giving SD rats these specialized diets their blood pressure (baseline) recordings were tested using the methods as described in the section “blood pressure measurements”. Rats were given free access to food and water. Rats with fructose water were given a supplemental water bottle with normal drinking water in it. All rats were housed three rats per cage in a temperature (22.7 ± 1.0 °C) and humidity (60 ± 10%). When rats were transferred to the metabolic cages, the rats maintained their specialized diet and were housed independently. Rats were given 24-hours to acclimate to their environment and any measurements within the first 24-hours was discarded. A 12-hours light and 12-hour dark cycle was maintained between 0700ET-1859ET and 1900ET-0659ET, respectively. Experimental operations were completed

within the Animal Care Facility at Michigan Technological University. All proper care and procedures of the rats was maintained throughout this study.

Prior to the diet treatment mean arterial blood pressure in conscious SD male rats were measured by non-invasive tail cuff using CODA© systems from Kent Scientific Corporation. Measurements for each male rat represents two separate recordings per week. Prior to the tail cuff recordings and specialized diets, all rats were acclimated to the animal holders for three consecutive days for 10 minutes each day. On the day of the recordings, each rat was place in the animal holder for 10 minutes before each blood pressure reading was recorded. For each blood pressure recording, the first five of the twenty cycles total were dismissed from this experiment during each run. After 3 weeks of measuring blood pressure and weights, rats were placed in the metabolic cages. Each rat has its own metabolic cage. 24-hours was allowed for the rats to acclimate to the metabolic cages and then measurements were recorded for the following three consecutive days. Rats maintained their specialized diet while in the metabolic cages. On the fourth day, rats were euthanized for brain tissue sampling as described below.

3.2 Paraformaldehyde infusion and brain sectioning

Eight-week-old male Sprague Dawley (SD) rats were divided into 4 groups and were fed a normal salt chow (NS: 0.4% NaCl), high salt chow (HS: 4% NaCl), NS with fructose (F: 20% fructose water), and HS plus fructose (HS+F:

4% NaCl and 20% fructose water) for 3 consecutive weeks. Adult male SD rats were euthanized by being injected with pentobarbatol (0.1 ml per 100g of body weight, 50 mg/kg). When the rats demonstrated a surgical level of anesthesia via toe pinch-response method, they were transferred into a surgical hood. Positioning the rat in a supine position, an incision in the integument and abdomen wall inferior to the rib cage was made. The diaphragm was completely cut in order to expose the pleural cavity. A continuous cut was made along the rib cage to the collar bone and repeated on the contralateral side. The sternum was clamped with the hemostat and placed over the rats head, and additional tissues connected to the heart was trimmed away to expose the beating heart. An incision to the right atrium was made and the 22-guage perfusion need was placed into the left ventricle towards the ascending aorta. After eliminating air bubbles from the tube, the 4% paraformaldehyde was infused into the rats system. Fixation tremors were observed within seconds of infusion and the rat was infused with PFA for a total of 10-15 minutes. The head from the rat was removed, and the brain was carefully removed from the skull. The brain remained in 4% paraformaldehyde in -4°C for 24-hours in a test tube and then was placed in 30% sucrose water for 3-4 days. Brain sections were cut no longer than 4 days in 30% sucrose using the cryostat machine (Leica Biosystems©). Brain cross-sections, specifically the paraventricular nucleus (PVN), were cut at 25μm starting from the optic nerve.

3.3 Immunostaining

Immunostaining of PVN GLUT5 was performed with the succeeding procedures: brain coronal sections (25um) containing the PVN was first washed in PBS for 3 times for 10 minutes total. After washing the slices with PBS, then the brain cross sections were incubated with 5% horse serum in PBS for 30 minutes. Then the cross sections were incubated with anti-GLUT5-antibody (Santa Cruze Biotechnology, CA, USA) with a 1:100 dilution in PBS containing 0.5% Triton C-100 and 5% horse serum for 3 days at 4ºC (trays were wrapped in parafilm to prevent dehydration). After 3 days, brain cross sections were washed with PBS 3 times for 10 minutes each. After the washing, the brain sections were then incubated with secondary antibody donkey Anti-Mouse IgG, Alexa Fluro® 488 (1:500 dilution) overnight at 4ºC while covered in aluminum foil to prevent photo-bleaching. In order to confirm that the observed antibody was staining, a negative control was performed for each antibody. The negative control was subjected to identical protocol for consistency similar to that of regular immunostaining. Instead, the negative control had PBS to ensure there was non-specific binding to other cellular sections that could possibly fluoresce. All sections were mounted in Vectashield© medium and glass slides and photos of each section were taken with Leica© DMIL microscope.

3.4 Fructose Assay

Eight-week-old male Sprague Dawley (SD) rats were divided into 4 groups and were fed a normal salt chow (NS: 0.4% NaCl), high salt chow (HS: 4%

NaCl), NS with fructose (F: 20% fructose water), and HS plus fructose (HS+F: 4% NaCl and 20% fructose water) for 3 consecutive weeks. Adult male SD rats were euthanized, blood serum and CSF was collected from each rat and 20uL of serum and CSF was used to determine the rat's true physiological measurement of fructose within itself after each specialized diet intake. Following the manufacturer's instructions data was obtained.

3.5 Real-time PCR (qPCR) Analysis of PVN GLUT5 mRNA Expression

Eight-week-old male Sprague Dawley (SD) rats were divided into 4 groups and were fed a normal salt chow (NS: 0.4% NaCl), high salt chow (HS: 4% NaCl), NS with fructose (F: 20% fructose water), and HS plus fructose (HS+F: 4% NaCl and 20% fructose water) for 3 consecutive weeks. Following the special diet treatment, rats were euthanized and their brains were removed. Paraventricular nucleus (PVN) tissue from the hypothalamus was punched. Punched PVN tissues were exposed to RNA isolation (RNeasy plus mini kit, Qiagen, CA, USA), and manufacture's guidelines were followed. qPCR was used to measure mRNA levels of GLUT5 using a primer or probe from the Real Time PCR System (Applied Biosystems, CA, USA). All data here was normalized to the housekeeping gene GAPDH mRNA.

3.6 Ion Chromatography

Eight-week-old male Sprague Dawley (SD) rats were divided into 4 groups and were fed a normal salt chow (NS: 0.4% NaCl), high salt chow (HS: 4%

NaCl), NS with fructose (F: 20% fructose water), and HS plus fructose (HS+F: 4% NaCl and 20% fructose water) for 3 consecutive weeks. Rats were then euthanized and blood, CSF and brains were collected. CSF and Serum samples were drawn for ion chromatography data. As previously described by Andrew Chapp 2018 et. al., serum and CSF samples were prepared to be tested in ion chromatography by adding 10uL of serum or CSF with 9.990 mL of ddH20 (18 MΩ), and then samples were vortexed and filtered from a syringe into sterilized vials [89]. Blood serum and CSF samples were then run by ion chromatography to determine their anions and cations [89].

3.7 Neuronal Culture Isolation

Primary neurons cultures were isolated using sterile technique from 24 hour old SD rats. Rat under 24 hours of age were euthanized and their whole brain was immediately separated apart from the cranium and vigilantly dropped into sterile DPBS solution. From the whole brain, neuron cells were carefully and quickly isolated and then plated into poly-L-lysis cultured dishes. It is known that, neuronal cultures contain mostly primary neuron cells and only about 5% astroglial cells. Primary neuronal cells medium that was used contained 1% penicillin and 10% horse serum in Dulbecco's Modified Eagle Medium© (DMEM). After two days from neuronal isolation, 50uL of ARC was added to 50mL of fresh medium, the cytosine arabinoside (ARC) sat in the isolated neuron cells for no more than 48 hours and then medium was changed back to fresh 1% penicillin plus 10% horse serum in DMEM (without ARC). Medium was not changed here

after this point, if needed, no more than 100uL of new medium was added one week after neuronal isolation. Primary neuron cells were maintained for 10-14 days old to ensure neuron cells had proper dendritic connections with other neuron cells or the plate.

3.8 Measurement of pro-inflammatory cytokines by Real-Time PCR in primary neurons cells

Primary neurons cultures were isolated using sterile technique from 24 hour old SD rats as described above. Cultures were made from Sprague Dawley (SD) rats whole brains containing the PVN. Momentarily, SD rats were euthanized and then the whole brain was immediately removed and tissues were combined. Neurons were disconnected with each other and promptly plated in poly-l-lysine coated dishes. Neuronal cultures contain nearly ~95% of neurons the remaining ~5% are astroglia cells. On the fourteenth day of neuron cells being plated in the sterile dish they were then subjected to treatments of fructose, sodium chloride (NaCl) and a combination of both at different concentrations. A control (no treatment what-so-ever) was always accounted for in each experiment.

3.9 Osmolarity

Eight-week-old male Sprague Dawley (SD) rats were divided into 4 groups and were fed a normal salt chow (NS: 0.4% NaCl), high salt chow (HS: 4% NaCl), NS with fructose (F: 20% fructose water), and HS plus fructose (HS+F: 4% NaCl and 20% fructose water) for 3 consecutive weeks. Rats were put in

metabolic cages for 4 nights then immediately after they were euthanized and blood, CSF and brains were collected. Urine, serum and CSF OSM was determined by the osmometer machine on the male SD rats for each rat group. Fresh ddH2O (18Ω) water was used in this osmometer. The machine was cleaned and standard solutions (290 mmol/kg and 1000 mmol/kg X3 runs) were used to calibrate the OSM machine prior to running any samples. Urine samples were diluted 10X in order for the machine to determine the value. CSF and blood serum were not diluted for determining OSM. (Data of osmolarity will not be provided for this thesis)

3.9 Data Analysis

Summary data is expressed in standard error of the mean (±SEM). Both in vitro and in vivo data were analyzed using a one-way Anova or unpaired student t-test. Evidences against the null hypothesis was considered statistically significant only if the P-value is < 0.05 indicated with an asterisk (*). A P-value > 0.05 would indicate weak evidence so that data would be considered failure to reject the null hypothesis.

4 Chapter 4: Results

4.1 A High Salt plus Fructose Diet Increases Blood Pressure in SD Rats

In order to prove that a 4% NaCl high salt diet plus 20% fructose water diet (HS+F) stimulated hypertension in SD rats and not on other specialized diets; eight-week-old male SD rats with the same ages were placed on either a NS or HS diet with and without fructose drinking water (NS, HS, F, HS+F) for 3 weeks. Blood pressure was measured via non-invasive tail-cuff plethysmography twice a week for 3 weeks **(Figure 4.1a)** and body weight was measured once per week for 3 weeks **(Figure 4.1b)**. After 3 weeks of diet treatment the rats were housed independently in metabolic cages. Food and water intake along with urine and fecal output was measured for 4 consecutive days **(Figure 4.1c)**. The first day of the metabolic data was discarded since the change of surroundings causes a rat to stress this day was reserved for acclimation to the metabolic cages. SD male rats on the NS (control) diet before the 3 weeks (91 mm Hg) compared to after 3 weeks (89 mm Hg) showed no statistical difference in mean arterial blood pressure (MAP) which is expected for control (**Figure 4.1a**). The SD male HS rat group 3 weeks before the HS chow treatment (93 mm Hg) showed no significant difference in MAP compared to 3 weeks after the HS diet treatment (96 mm Hg) (**Figure 4.1a**) . The SD male F rat group 3 weeks before the 20% fructose drinking water treatment (97 mm Hg) and after 3 weeks of 20% fructose in drinking water treatment (98 mm Hg) showed no statistical difference in MAP as well (**Figure 4.1a**). Surprisingly, the HS+F SD

male rat group (HS+F baseline: 98 mm Hg) showed a significant statistical difference in MAP after the 3 weeks of a HS chow combined with 20% fructose in drinking water (126 mm Hg) (**Figure 4.1a**).

In summary, after three weeks of special diets SD rats on a NS diet (89 mm Hg), HS alone diet (96 mm Hg), and F alone diet (98 mm Hg) showed no change in MAP. However only when the combined diet HS+F (127 mm Hg) was given there was an increase in MAP. The SD rat group for NS has a significant (P<0.000001) increase in MAP by a total of ~36 mmHg compared to itself prior to receiving a special diet treatment (Baseline of NS rats: 91±3 mmHg vs. HS+F rats after 3 weeks of diet 127±6 mmHg) **(Figure 4.1a)**.

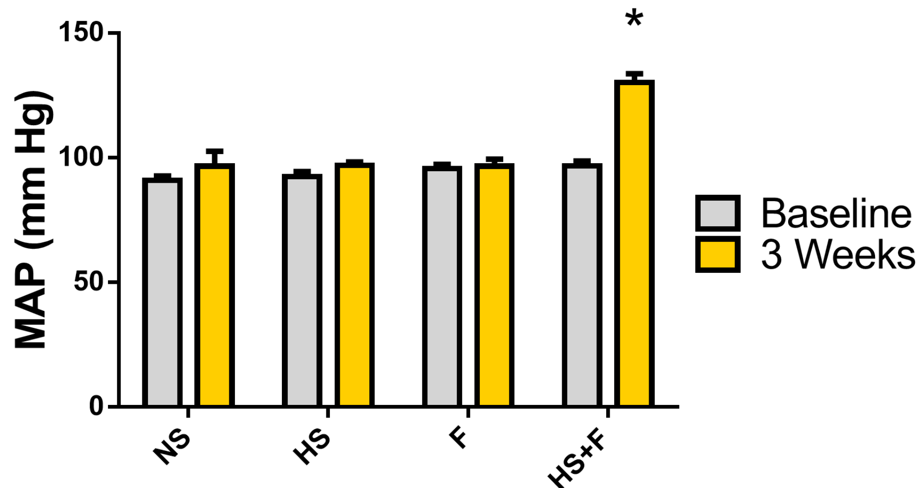


Figure 4.1a Conscious mean arterial blood pressure (MAP) for Sprague Dawley (SD) rats on a normal salt (NS; 0.4% NaCl and normal water; n=15), high salt (HS; 4% NaCl and normal water; n=15), fructose (F; normal chow and 20% fructose water, n=15), and high salt plus fructose (HS+F; 4% NaCl chow plus 20% fructose water, n=15) diet before and after 3 weeks of diet treatment.

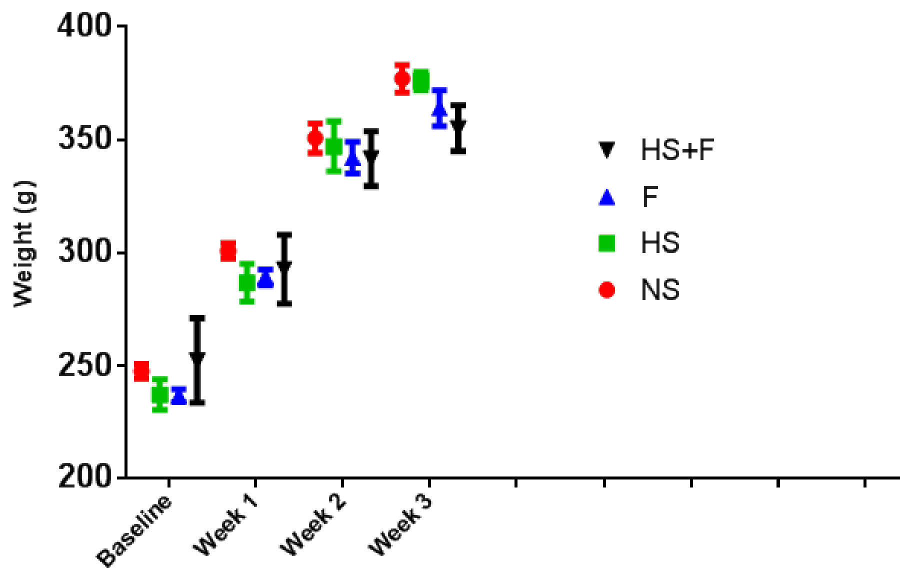


Figure 4.1b Rat's body weights were measured for each rat given a normal salt (NS; 0.4% NaCl and normal water, n=5), high salt (HS; 4% NaCl and normal water n=5), fructose (F; normal chow and 20% fructose water; n=5), and high salt plus fructose (HS+F; 4% NaCl chow plus 20% fructose water, n=5) diet before and after 3 weeks of diet treatment.

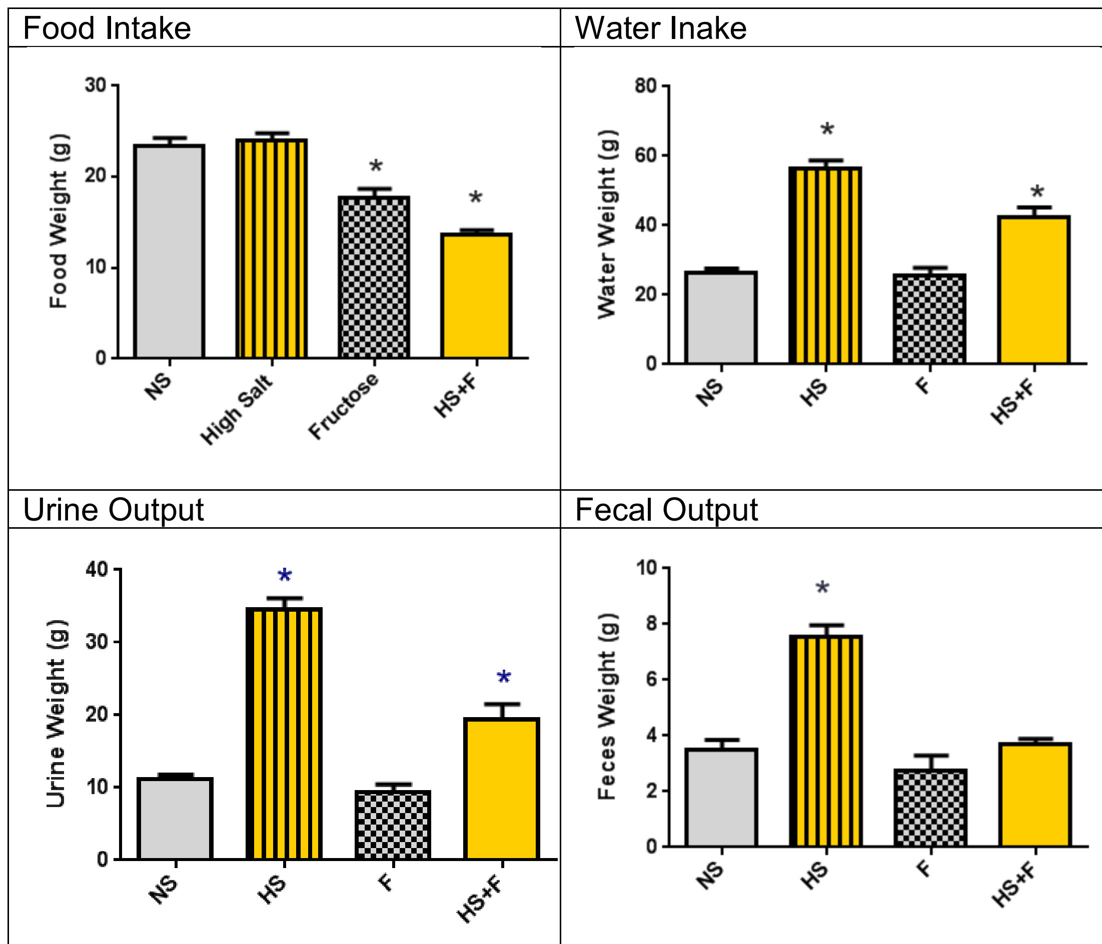


Figure 4.1c After 3 weeks of each rat given a normal salt (NS; 0.4% NaCl and normal water), high salt (HS; 4% NaCl and normal water), fructose (F; normal chow and 20% fructose water), and high salt plus fructose (HS+F; 4% NaCl chow plus 20% fructose water) diet they were subjected to metabolic cages. Rat's food and water intake along with urine and fecal output was measured every 24 hours for 4 days. *P<0.05, student paired t test. One Way Anova performed on urine and water intake P<0.005. (n=15 per each group).

4.2 Immunostaining

To provide results that GLUT5 was demonstrated to be expressed in the rats PVN brain area and plays a role in whether a HS+F diet alters GLUT expression in SD rats and age matched those of the NS. Eight-week-old male SD rats were fed either a NS or HS diet with and without fructose drinking water

(NS, HS, F, HS+F) for 3 weeks. After 3 weeks, SD male rats were euthanized and whole brains were taken and immediately frozen in liquid nitrogen. Brain slices were cut on a cryostat machine at 20µm thick. PVN area were used for immunostaining with GLUT5. All groups were stained (not shown). HS alone diet and F alone diet groups of brain GLUT5 immunostaining did not show any expression, all images were dark so they are not revealed in the following figure. GLUT5 is highly expressed in the high salt plus fructose treatment of the brain hypothalamic PVN tissue **(Figure 4.2 c,d)**.

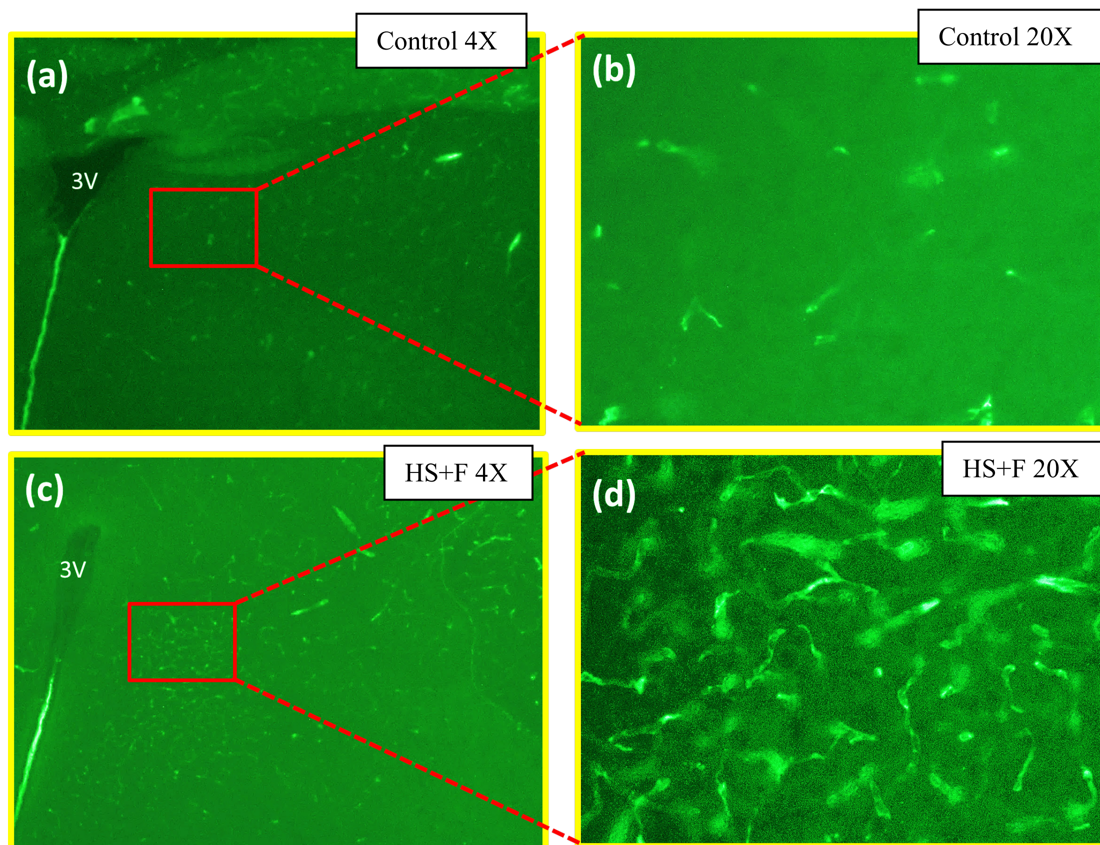


Figure 4.2 The area of the PVN surrounding the third ventricle (3V) is shown by 4X magnification (a) of the PVN for the NS (control group), and (b) demonstrates the area in the box at 20X magnification for the NS group. The area of the PVN

sounding the 3V is shown by 4X magnification (c) for the HS+F group and (d) shows the area of the box at 20X magnification of the HS+F group.

4.3 Real Time Polymerase Chain Reaction of GLUT5

To further provide evidence that GLUT5 is expressed in the brain hypothalamic PVN we performed Real Time PCR (qPCR) of the PVN area. Eight-week-old male SD rats were fed either a NS or HS diet with and without fructose drinking water (NS, HS, F, HS+F) for 3 weeks and their age matched that of the previous rats. The SD male rats were euthanized after their special diet treatment (NS, HS, F, HS+F), PVN tissue was punched out and qPCR was performed to evaluate the neuronal maker for fructose which was GLUT5 mRNA levels. SD rats on a NS, HS and F alone diet showed not significant difference in the change of PVN mRNA levels of GLUT5 (NS: XX, HS: XX, F: XX) compared to HS+F SD eight-week-old male rats ($P<0.05$). GLUT5 was confirmed to be upregulated in the brain hypothalamic PVN tissue for the HS+F rats compared to the NS, HS, and F rat groups **(Figure 4.3)**.

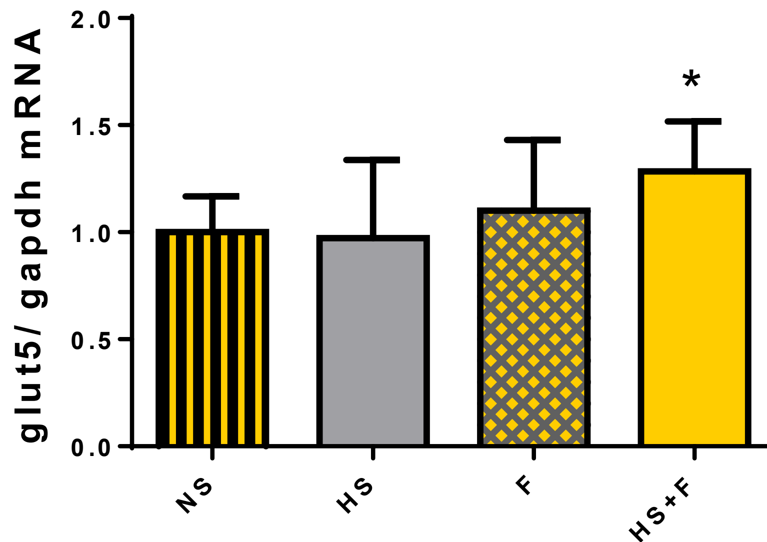


Figure 4.3 Real Time PCR (qPCR) data for four different groups: NS (control), HS (4%NaCl chow, normal drinking water), F (normal chow, 20% fructose drinking water), HS+F (4%NaCl chow with 20% fructose drinking water), respectively. GLUT5 is upregulated in HS+F group. (NS: n=5, HS: n=3, F: n=2, HS+F: n=4)

4.4 Fructose Assay

To determine the physiological concentration of fructose from *in vivo* of the rat's blood serum and CSF fructose assay kit was utilized. This was done to ensure similar concentrations were used for *in vitro* studies. Eight-week-old male SD rats were fed either a NS or HS diet with and without fructose drinking water (NS, HS, F, HS+F) for 3 weeks and their age matched that of the previous rats. The SD male rats were euthanized after their special diet treatment (NS, HS, F, HS+F), and blood serum, CSF and whole brains were collected. Manufactures instructions were followed for the fructose assay kit. Fructose concentrations in blood serum increased to 359μM in the fructose group, and to 409μM in the

HS+F group (P<0.05) compared to NS group of 253μM **(Figure 4.4a)**. CSF fructose decreased in the F group to 230μM (P<0.05) compared to the NS group of 290uM **(Figure 4.4b)**.

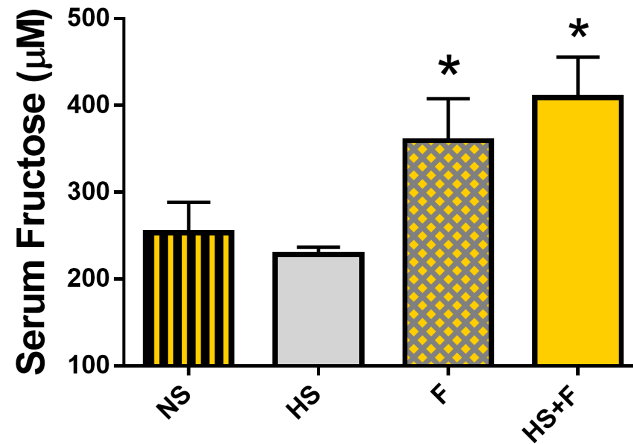


Figure 4.4a Blood serum of 8-week-old Sprague Dawley (SD) rats were withdrawn from four different groups: NS (control), HS (4%NaCl chow, normal drinking water), F (normal chow, 20% fructose drinking water), HS+F (4%NaCl chow with 20% fructose drinking water), respectively. Serum concentration increased in the F and HS+F group. (NS: n=5, HS: n=3, F: n=4, HS+F: n=5).

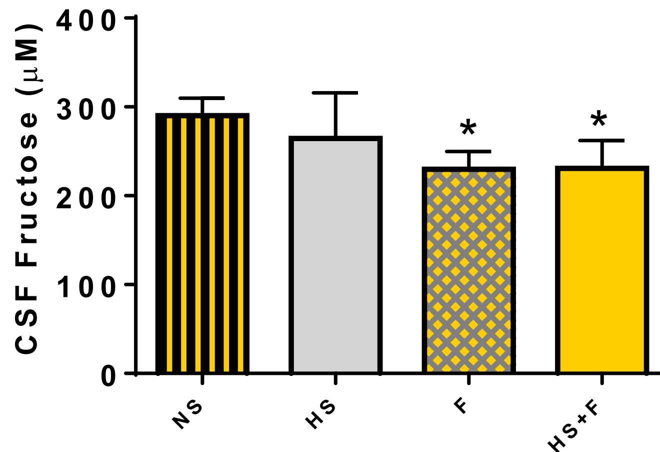


Figure 4.4b CSF of 8-week-old Sprague Dawley (SD) rats were withdrawn from four different groups: NS (control), HS (4%NaCl chow, normal drinking water), F (normal chow, 20% fructose drinking water), HS+F (4%NaCl chow with 20% fructose drinking water), respectively . CSF fructose decreased in the F group compared to the NS group. (NS: n=5, HS: n=3, F: n=4, HS+F: n=5).

4.5 Ion Chromatography

To determine the anions and cations of the SD male rat's CSF, ion chromatography was utilized for this purpose. After 3 weeks of each SD male rat given a normal salt (NS; 0.4% NaCl and normal water), high salt (HS; 4% NaCl and normal water), fructose (F; normal chow and 20% fructose water), and high salt plus fructose (HS+F; 4% NaCl chow plus 20% fructose water) diet their CSF sodium, acetate, lactate, magnesium, potassium, and calcium was measured **(Table 1)**. A significant difference water determined ($P<0.05$) between sodium and the control/NS group (150.47 mM) vs. the HS+F (160.6 mM) group **(Figure 4.5a)** and there was also a statistically significant difference ($P<0.05$) regarding

acetate levels in the CSF between control (0.0715 mM) vs. F (0.1007 mM) and control (0.0715 mM) vs. HS+F (0.1945 mM) groups **(Table 1)**.

Averaged CSF Electrolytes for Eight-Week-Old Male SD Rats After 3 Weeks of A Special Diet Treatment Give							
	Cations				**Anions**		
Diet Group	Calcium (mM)	Potassium (mM)	Sodium (mM)	Magnesium (mM)	Acetate (mM)	Lactate (mM)	Chloride (mM)
NS	3.1	2.5	150.5	2.1	0.0715	2.6	119.3
HS	3.5	2.3	158.1	2.2	0.1551	2.5	124.3
F	3.4	2.1	155.1	1.9	*0.1007	2.1	120.9
HS+F	2.9	2.1	*160.6	2.1	*0.2	2.8	125.1

Table 1 Eight-week-old male Sprague Dawley (SD) rats were divided into 4 groups and were fed a normal salt chow (NS: 0.4% NaCl), high salt chow (HS: 4% NaCl), NS with fructose (F: 20% fructose water), and HS plus fructose (HS+F: 4% NaCl and 20% fructose water) for 3 consecutive weeks. Rats were euthanized and CSF was withdrawn from the rats. CSF was tested for calcium, potassium, sodium, magnesium, acetate, lactate, and chloride. *P<0.05 compared to the control (NS) group. (n=5 per each group).

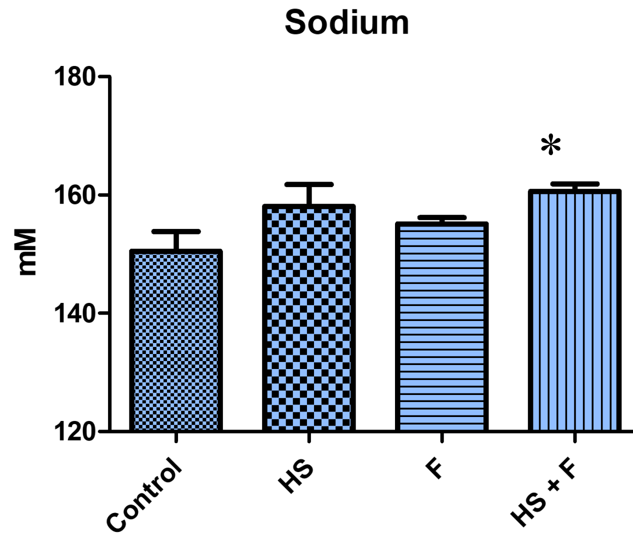


Figure 4.5a Eight-week-old male Sprague Dawley (SD) rats were divided into 4 groups and were fed a normal salt chow (NS: 0.4% NaCl), high salt chow (HS: 4% NaCl), NS with fructose (F: 20% fructose water), and HS plus fructose (HS+F: 4% NaCl and 20% fructose water) for 3 consecutive weeks. Rats were euthanized and CSF was withdrawn from the rats. Sodium in cerebrospinal fluid (CSF) was obtained by ion chromatography. Control (NS): 150.4739 mM; HS: 158.0899 mM; F: 155.1233 mM; HS+F: 160.6339 mM. *$P<0.05$ compared to the control (NS) group. (n=5 per each group).

4.6 Real Time PCR for Pro-inflammatory Cytokines of Brain Neurons

In order to determine the inflammatory responsible for inducing pre-inflammatory cytokines during a high salt and fructose treatment, primary neuronal cultures from the whole brain of less than 24-hour old SD male rats were incubated with high salt, fructose, and a combination of high salt and fructose treatment for 6 hours. Real time PCR was performed to measure mRNA levels of *NfKB1* (**Figure 4.6a**) and IL6 (**Figure 4.6b**).

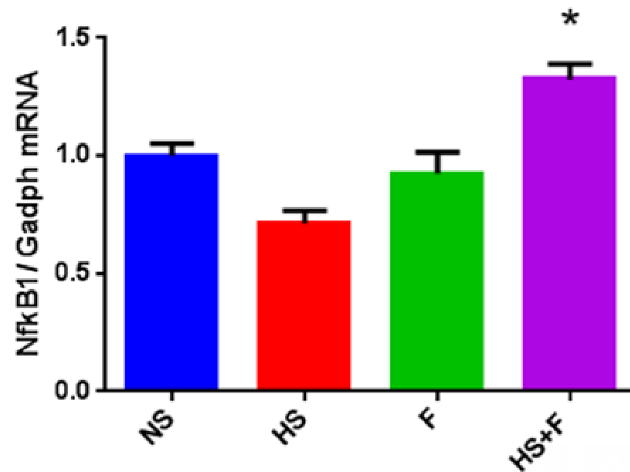


Figure 4.6a Primary neuron cells were isolated from SD rats within 24 hours of birth. 12-day-old neurons were treated with different doses of salt and fructose for 6 hours and then RNA was extracted and then converted to cDNA followed by RT PCR. HS represents 5mM of NaCl treatment, F indicated 5mM of fructose and HS+F indicates 5mM of high salt combined with 5 mM of fructose.

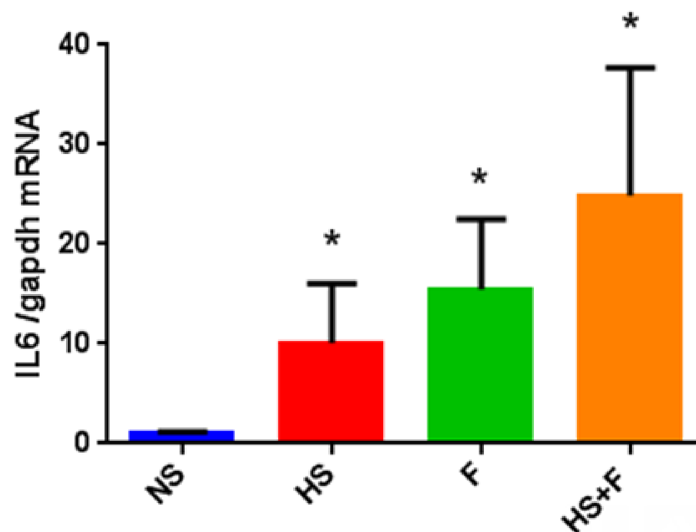


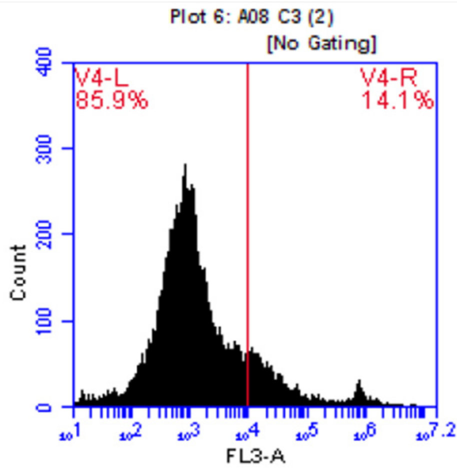
Figure 4.6b Primary neuron cells were isolated from SD rats within 24 hours of birth. 12-day-old neurons were treated with different doses of salt and fructose for 6 hours and then RNA was extracted and then converted to cDNA followed by RT PCR. HS represents 5mM of NaCl treatment, F indicated 5mM of fructose and HS+F indicates 5mM of high salt combined with 5 mM of fructose.

4.7 Flow Cytometry of Primary Neuron Cells

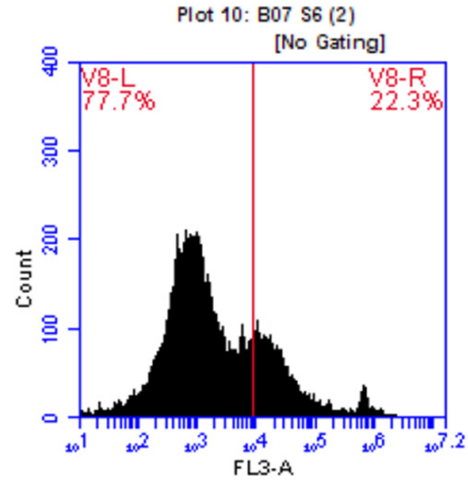
Isolated primary rat neuronal cells demonstrated apoptosis as a reaction to fructose and/or sodium chloride (NaCl) treatments. Incubation of brain neurons isolated from neonatal SD rats with 200μM (17.2%, $P<0.05$) and 1mM (22.5%, $P<0.005$) of a 6 hour fructose treatment stimulated neuronal apoptosis **(Figure 4.+-7)** compared to the control (14.1%). Isolated neurons were also subjected to a 6 hour treatment of NaCl alone (19.1%, no significant difference), and a combination of 200μM fructose + 5mM NaCl (22.3%, $P<0.005$) **(Figure 4.7)**. Finally, we tested 1mM of fructose + 5mM NaCl (24.3%, $P<0.005$) on primary rat neuronal cells **(Figure 4.7)**. This study demonstrated that HS+F diet decreases CSF fructose concentration, and that the physiological amount of fructose concentration is shown to stimulate neuronal apoptosis in brain neurons, and increases PVN GLUT5 expression, which may result in hypertension.

Flow Cytometry Data from Primary Isolated Neuron Cells Treated with Either NaCl, Fructose and Both

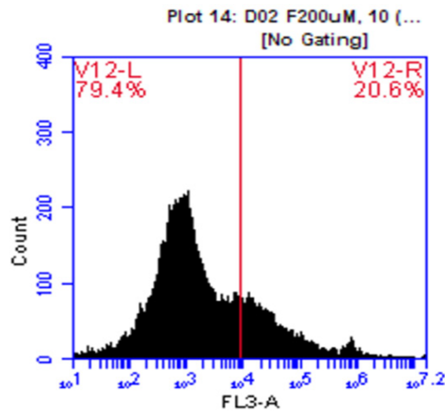
(a)



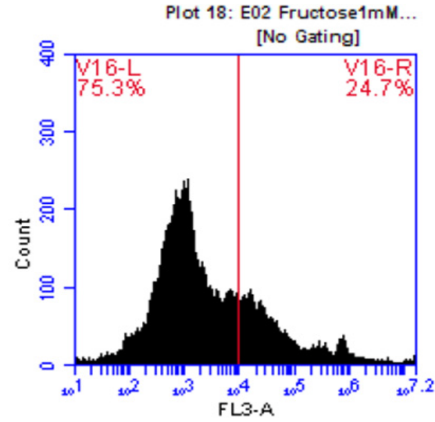
(b)



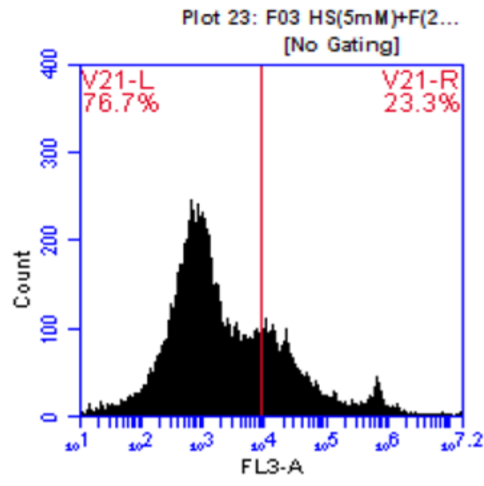
(c)



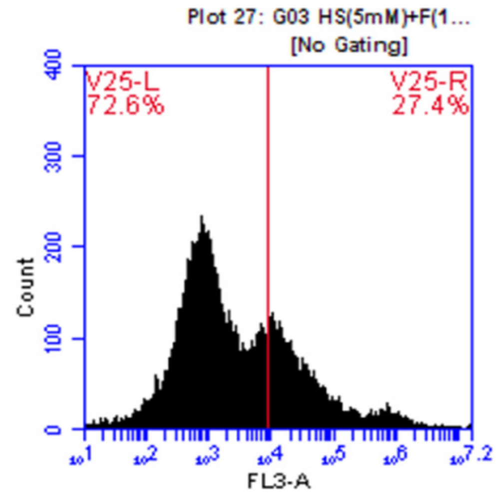
(d)



(e)



(f)



(g)

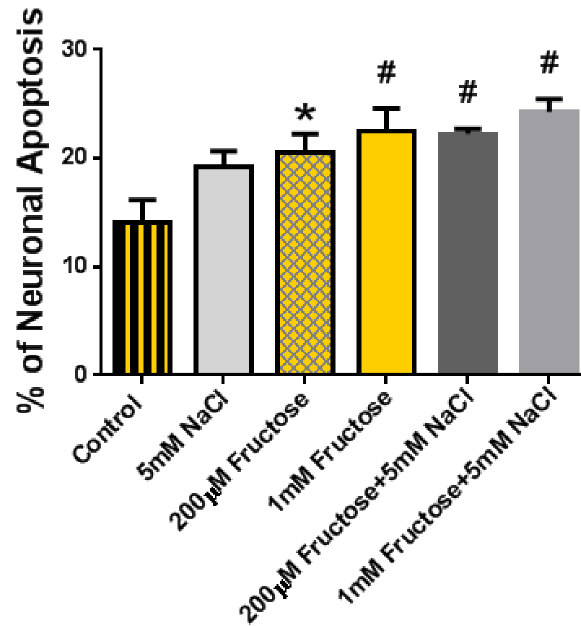


Figure 4.7 Primary neuron cells were isolated from SD rats within 24 hours of birth. 12-day-old neurons were treated with different doses of fructose for 6 hours and then stained with propidium iodine (PI) and flow cytometry was performed. (a) Represents the control group, (b) is the 5mM of NaCl treated neuron cells for 6 hours. (c) is the 200μM fructose treated neurons for 6 hours, and (d) is 1mM of fructose treatment for 6 hours. (e) Represents the combination of 6 hour treatments 200μM Fructose+5mM NaCl and (f) is the combined 6 hour treatment of 1mM fructose+5mM NaCl. (g) Is the percentages of neuronal apoptosis of from each treatment depicted in (a-f). $^*P<0.05$, $\#P<0.005$. (n=4 per each group).

5 Chapter 5: Discussion

5.1 Main Idea

The central nervous system (CNS) plays an imperative role in blood pressure regulation and has been thought to be the primary role of fructose-induced hypertension. The sympathetic nervous system (SNS) plays a more important role in controlling blood pressure (BP). More specifically, the pre-sympathetic neurons with efferent projections to the brainstem areas are the central regulator of the SNS. The pre-sympathetic neurons contribute to the main role in blood pressure regulation and sympathetic nervous system activity (SNA). However, the key area that has control of BP and SNA is the paraventricular nucleus of the hypothalamus (PVN). The PVN includes monosynaptic, and glutamatergic projections to the rostral ventrolateral medulla (RVLM) which impacts sympathoexcitation.

Taking everything into consideration evidence suggests that a high salt and fructose diet alters neuronal cells by stimulating neuronal apoptosis and fructose stimulates salt uptake in brain CSF resulting in upregulation of GLUT5 in brain paraventricular nucleus (PVN) of the hypothalamus. Since the PVN is a key pre-sympathetic region with regulatory control over BP and SNA then we have shown here that the high salt and fructose diet combined is the main contributor to BP regulation and responsible for the increase in BP.

Evidence that the PVN tissue expressed GLUT5 demonstrates that a combined high salt and fructose diet may play a more central role in of cardiovascular function contributing to hyperactivity in the PVN area resulting to neurogenic hypertension[90]. The mechanism of fructose induced hypertension is not known. This study is also the first to determine that elevated sodium levels is seen in a SD male rats after three weeks of diet treatment of 4% NaCl combined with 20% fructose drinking water (HS+F) relative to the control; NS, normal salt plus normal drinking water.

First, MAP is increased by a total of 37 mmHg in eight-week-old SD male rats after just 3 weeks of a HS+F diet compared to NS rats. Second, the expression of GLUT5, a fructose transporter, is expressed in brain PVN and quantified by real time PCR demonstrated upregulation of GLUT5 in PVN tissue. Third, brain hypothalamic primary neurons exposed to fructose plus sodium uptake in brain neurons results in apoptosis in just 6 hours of treatment. Finally, CSF sodium was increased is those rats who were given a 20% fructose diet and even a higher increase in sodium from the rats who received HS+F diet treatment. Although, CSF fructose concentrations of the HS+F rats decreased compared to control may be an indication that a moderate fructose consumption alone may stimulate sodium uptake in CSF of adult Sprague Dawley (SD) male rats. What is interesting is that Per Enshe Jiang et. al. 2018, confirmed that CSF sodium concentrations of Dahl salt sensitive rats was elevated when given a HS 4% NaCl diet (158 mM) [91] compared to this study when SD rats when fed a HS+F

diet (160 mM). As you can see the CSF sodium concentrations of the Dahl salt sensitive rats compares to the HS+F SD rats. This further indicates that the combined high salt and fructose diet stimulates salt sensitivity linking to an increase in mean arterial blood pressure through means of the PVN. Since the PVN includes the monosynaptic and glutamatergic projections to the rostral ventrolateral medulla (RVLM) which impacts sympathoexcitation. Simply put, this combined high salt and fructose diet creates the abnormalities of the central regulation of SNA which dysregulates the pre-sympathetic neurons. Furthermore, we have sought here that salt changes the GLUT5 protein transporter in the PVN under a high salt and fructose combined diet. The changes made in the GLUT5 protein transporter may be the contribution to inflammation and neuronal apoptosis further linking to hypertension.

Collectively, our results demonstrated that fructose may cause hypertension through the mechanism of neuronal apoptosis in the PVN and that a HS+F diet contributes to salt sensitivity in SD rats. The PVN, protected by the blood brain barrier, is the main site for neuroendocrine and automatic function [92, 93]. Expression of GLUT5 after high salt and fructose diet is unknown to be widespread in the entire body, but the PVN area demonstrated GLUT5 expression which may be the missing link when contribute to blood pressure regulation during fructose-induced hypertension.

5.2 Summary

Here we have demonstrated that fructose and salt synergistically create hypertension in eight-week-old male Sprague Dawley (SD) rats. Furthermore, we have demonstrated that an increased expression of GLUT5 in the PVN of SD rats who were given a 4% high salt and 20% fructose drinking water (HS+F) diet for 3 weeks. This suggests that a HS+F diet stimulates the uptake of fructose into neuronal cells which results in the increase of intracellular fructose and an increase in CSF sodium concentrations. The increase in CSF sodium concentrations is comparable to Dahl salt sensitive rats on a high salt diet [91]. The upregulation of nuclear factor NS-kappa B (*NFkB1*) in the PVN of the HS+F rats further demonstrates that a fructose and high salt diet is linked to inflammation and inappropriate immune cell damage in the CNS.

To review the data, this study demonstrated that a just 3 weeks of a HS+F diet increased MAP in SD male adult rats. Chow (food) intake decreased in F and HS+F SD rat groups. Water intake and urine output increased significantly in HS and H+F rat groups only which is hallmark for diagnosing hypertension. Rat's fructose concentration increased in serum in F and HS+F but decreased in CSF for F and HS+F SD male rat. HS+F SD male rat group increased GLUT5 expression in the brain hypothalamus and in the PVN area. Increased mRNA expression of GLUT5 in the PVN area was demonstrated by real time PCR compared to NS, HS, and F groups. CSF sodium increased in HS+F group which is comparable to Dahl salt sensitive rats on a high salt diet.

5.3 Outlooks

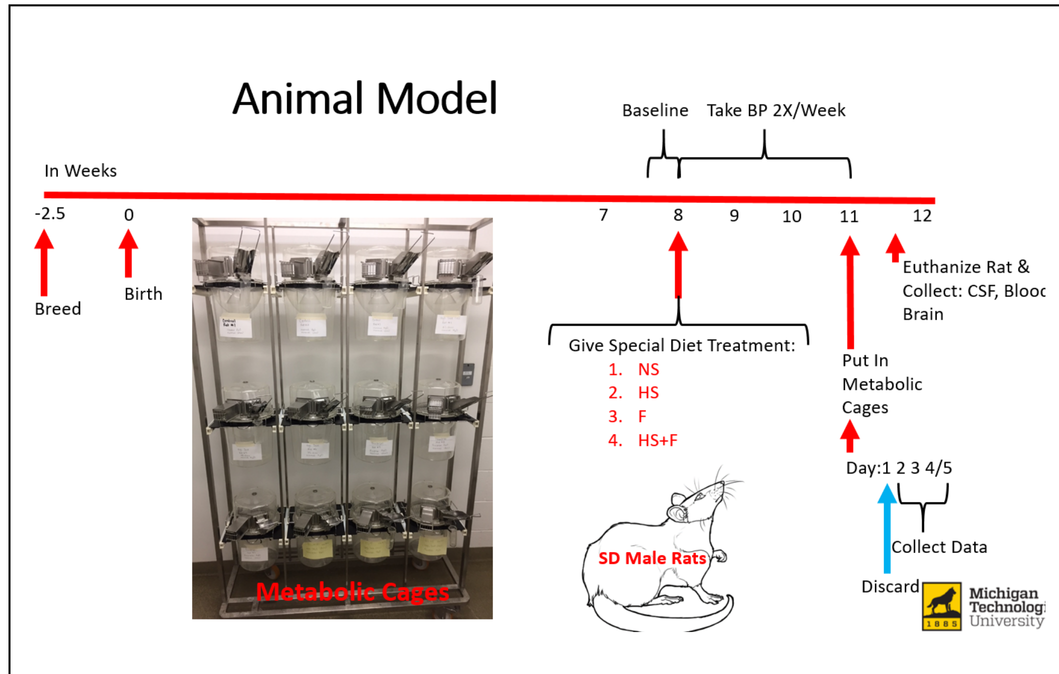
This research sheds light on the gap in knowledge between the connection of fructose and salt intake and hypertensions within the CNS. Those with hypertension or pre-hypertension and metabolic syndrome are at risk for stroke, cardiovascular disease, and heart failure. Increase salt and fructose combined intake contributes to both hypertension especially in metabolic syndrome patients. It is of great interest for those with pre-hypertension to decrease intake of fructose and salt consumption. For further treatment and to reduce the number of cardiovascular deaths the salt sensitive population should watch both salt and fructose intake. This study indicates that a combined salt and fructose diet increase CSF acetate and mediates hypertension through neuronal apoptosis and increasing pro-inflammatory cytokines.

5.4 Future Studies and Restrictions

Brain PVN punch tissue is not exact, and obtaining a portion of the other brain areas may occur. The blood pressure tail cuff method was used, but to better this study the renal transducers should be implanted in the rats to determine a more accurate blood pressure recording. Other future studies include an interperotoneal injection of fructose to determine a more acute increase of lactate response in the cerebral spinal fluid (CSF) by measurements done from ion chromatography.

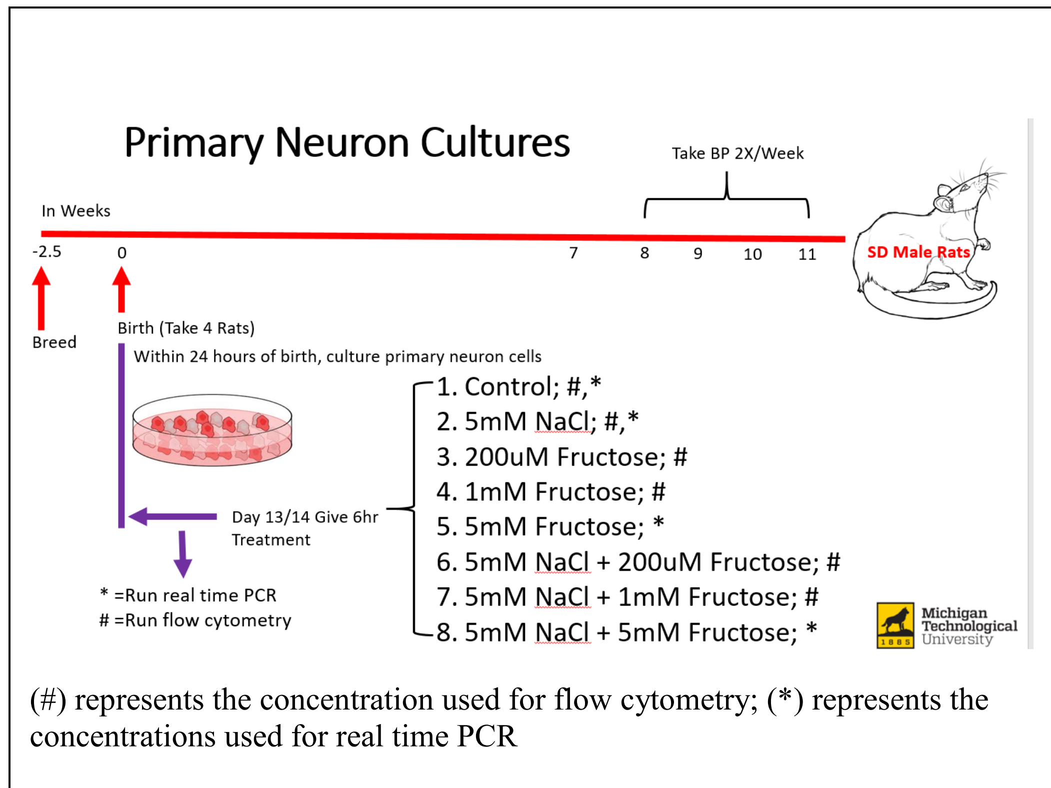
6 Chapter 6: Supplementary Tables and Diagrams

6.1 Animal Model Timeline for Diet Treatments and Metabolic Cage Measurements



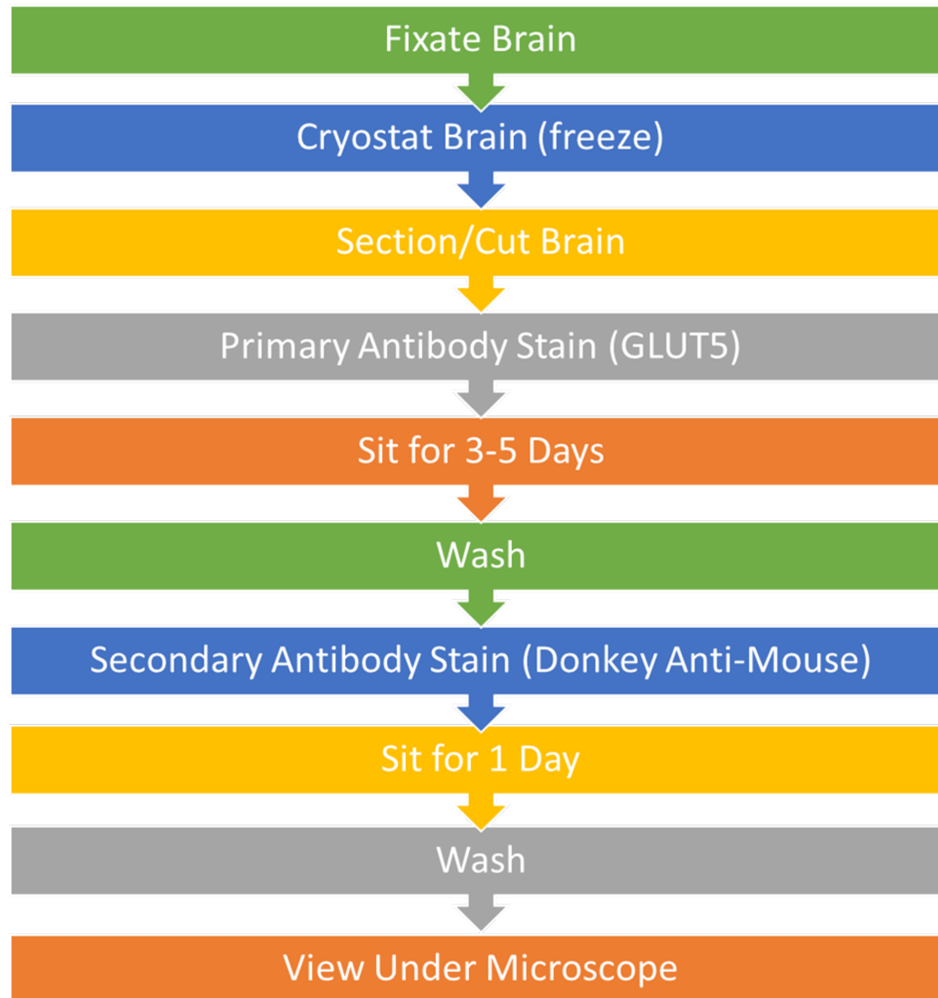
6.2 Blood Pressure Apparatus

6.3 Primary Cultured Neuron Cells for RT PCR and Flow Cytometry



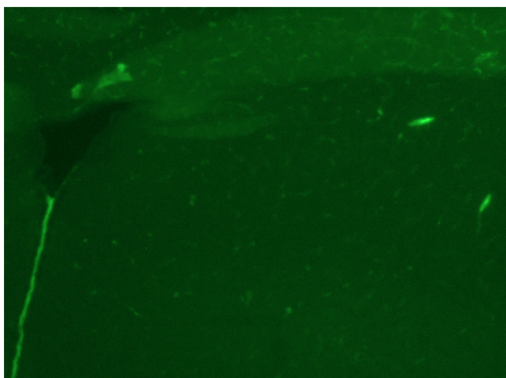
(#) represents the concentration used for flow cytometry; (*) represents the concentrations used for real time PCR

6.4 Immunostaining Flow Diagram

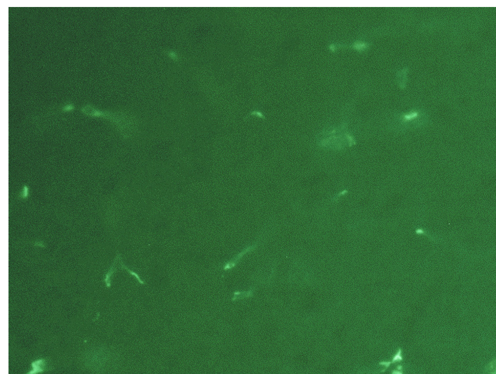


6.5 Co-Immunostaining Brain Section Pictures

Control X4

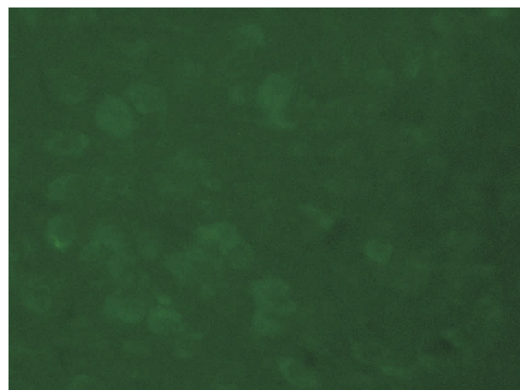


Control X20

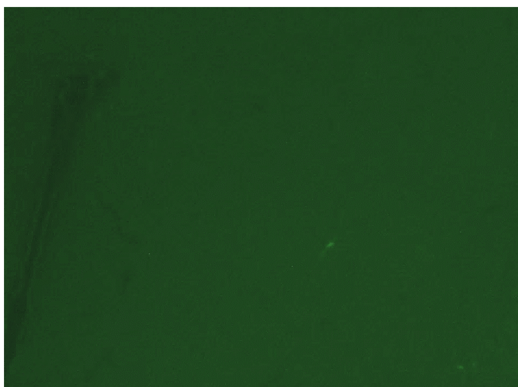


HS 4X

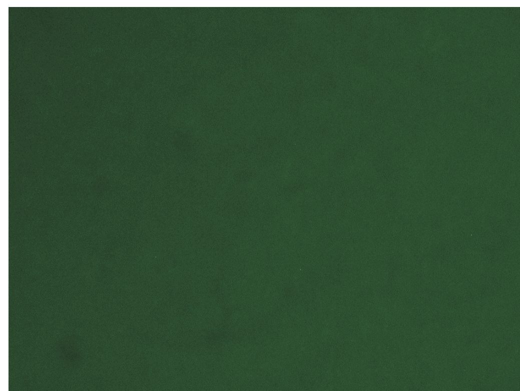
HS 20X



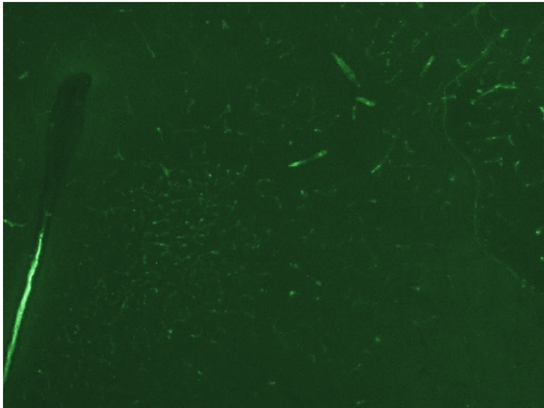
Fructose 4X



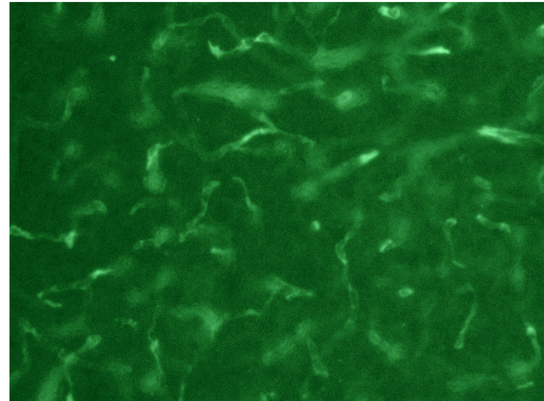
Fructose 20X



HS+F 4X



HS+F 20X



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