THE IMPACT OF THE ANTI-INFLAMMATORY DRUG PLX3397 ON HYPOTHALAMIC MICROGLIA ACTIVATION AFTER HIGH FAT FEEDING AND THE EFFECTS OF PRENATAL SURGICAL STRESS FOLLOWED BY HIGH FAT FEEDING ON THE DEVELOPMENT OF OBESITY

by

BRENT NORWOOD

(Under the Direction of Krzysztof Czaja and Sheba Mohankumar)

ABSTRACT

Obesity is a major health concern worldwide. Many therapies have been developed in an attempt to combat its increasing prevalence. High fat diet and stress have been shown to play a major role in the development of obesity. The effect of high fat diet in the brain and its downstream effect throughout the body is not completely understood. The inflammation in the central nervous system that occurs after high fat feeding has the potential to disrupt metabolic homeostasis and neurotransmission in the brain. Anti-inflammatory drugs are readily available on the market and may be an easy therapy to treat the neuroinflammation that results from consumption of a high fat diet. Our study found that an anti-inflammatory drug does in fact suppress microglia activation, therefore decreasing brain inflammation. In addition to the simple consumption of high fat diet, stress is a compounding factor that plays a major role in the development of obesity. Fetal programming is a concept that is now being used in many studies in an attempt to understand the factors that might predispose offspring to developing diseases like obesity in adulthood. Prenatal stress followed by postnatal high fat diet exposure may be a very effective paradigm to

study the mechanisms involved that promote obesity in the offspring. A diet induced obese animal model that is genetically predisposed to obesity along with its lean counterpart was used in these studies. This model was used to study the complex interaction between genes, prenatal stress, high fat diet, and sex in the development of obesity. Our study found that maternal stress did in fact program the offspring to develop obesity and modulate other metabolic factors after being challenged with high fat diet. The impact of these interactions on neurotransmitter systems and hormones is presented and our study shows significant differences between the genetic, prenatal stress, diet, and sex groups. Preliminary understanding of brain neurotransmission in this context might help us to draw a more complete picture of the changes associated with a high fat diet-induced inflammatory state.

INDEX WORDS: High fat diet, Hypothalamus, Microglia, Obesity, Diet-induced obesity, Dietary resistant, Prenatal programming, Stress

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DEDICATION

I would like to dedicate my dissertation to my parents and grandparents that instilled in me a strong work ethic and a drive to pursue my goals. To my wife, Laura for all of her sacrifices and support to help me get to this point. To the two greatest gifts of my life so far:

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER 1
INTRODUCTION

1.1 Statement of Purpose

As the prevalence of obesity has increased dramatically in the past two decades and in recent years, further investigation is warranted for the health of populations around the world. The increased consumption of a high-fat diet (HFD) may be one of the major culprits for the development of obesity by causing an inflammatory state within the brain. The inflammatory state within the brain is indicated by microglia activation. Anti-inflammatory drugs are available that may play a role in preventing or suppressing microglia activation in the brain, but this mechanism is not fully understood. Therefore, we investigated the impact of an anti-inflammatory drug (Pexidartinib; PLX3397; PLX) in combination with HFD on inflammation in regions of the brain involved in feeding regulation. We used rats fed standard chow diet (CD) and HFD in combination with and without PLX to study the effect of PLX on microglia activation within specific hypothalamic nuclei related to feeding regulation as well as the nucleus tractus solitarius (NTS).

Previous studies have focused on many mechanisms that may be involved in the development of obesity such as HFD, high sugar diet (HSD), other combinations of high fat and high sugar diets (high energy density diets; HED), nutritional deficiencies in the diet, deficiencies in the diet during pregnancy, prenatal stress, prenatal alcohol exposure, and prenatal exposure to endocrine disruptors. These factors induce “prenatal programming” of the fetus as a result of which there are molecular and cellular alterations at the tissue level which leads to the development of obesity and susceptibility to the

development of metabolic syndrome in adulthood [1-3]. Within the brain, there are chemical messengers whose activities, when disrupted, may also play a role in the development of obesity. The three main monoamine neurotransmitters of interest are norepinephrine (NE), dopamine (DA), and serotonin (5HT). These neurotransmitters (NTs) are involved in the regulation of multiple body functions and have been studied for other disorders/mental illnesses such as depression and anxiety. As these NTs have been shown to modulate mood, and body functions such as gastrointestinal control, and hormone secretion to name a few, they have been implicated to play a role in feeding behaviors, but this is not fully understood[4]. Moreover, prenatal stress has been reported to play an important role in the development of obesity [5, 6]. However, the role played by NTs and hormones in this phenomenon is not well understood. Therefore, we studied the impact of prenatal stress in combination with maternal HFD exposure on food intake and body weight as well as the changes in monoamine NT levels in the brain. In addition, we used a diet-induced obese and dietary resistant (DIO/DR) rat model that is genetically predisposed or resistant to obesity respectively in our experiments to understand if genetic predisposition further aggravates the effects of prenatal stress.

The overall aim of this dissertation is: 1) to investigate the effect of PLX on inflammation in the brain in response to HFD promoting obesity, 2) to investigate the effects of prenatal stress in combination with maternal HFD exposure on metabolic functions promoting obesity in DIO/DR offspring, and 3) to understand the impact of prenatal stress and HFD exposure on monoamine NTs in promoting obesity in DIO/DR rats. The first aim uses a mechanistic approach to understand the role of microglia in promoting obesity since a number of recent studies have demonstrated their activation after HFD

feeding [7, 8]. The second and third aims are correlative as the paradigm used for inducing prenatal stress is relatively untested in the context of obesity and the involvement of NT activity in this paradigm of prenatal stress has not been investigated before. This research will give insights into the neuroinflammatory, metabolic, and neurochemical mechanisms involved in the development of obesity.

1.2 Obesity

Obesity is a widespread condition impacting humans globally. The National Health and Nutrition Examination Survey (NHANES) estimated that the prevalence of obesity in the US had increased from 30.5% in 2000 to 41.9% in 2021. During the same time frame, the prevalence of severe obesity increased from 4.7 to 9.2% (Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS) [9]. Historically, obesity has been measured using the body mass index (BMI). BMI levels have trended in the same pattern as obesity indicating that it is a reliable method of measuring obesity rates. BMI is recorded as weight in kilograms divided by height in meters squared [10]. The hallmark of obesity is the chronic imbalance of energy homeostasis leading to an accumulation of body fat. Obesity is multifactorial in origin with genetics and other environmental factors playing a role in its development and many of these factors are not fully understood.

The complex nature of the cause of obesity is probably why it is so difficult for individuals to overcome this condition. Some of the potential factors causing obesity are a sedentary lifestyle, consumption of diets high in fat/sugar, or being subject to psychosocial stress to name a few [11, 12]. Besides the fact that obesity is increasing in epidemic proportions, it has become a major concern because of the severe health risks that it

promotes and the staggering burden that it places on the economy. Some of the health risks include heart disease, stroke, type-2 diabetes, and cancer which are the leading causes of death in the United States for preventable diseases, therefore leading to a shortened lifespan [13]. Sadly, obesity is a preventable and even perhaps a reversible disease. There are numerous resources that have been developed to help individuals combat this disease, however, the sustained increase in the prevalence of the disease demonstrates the need for better, more effective interventions [14, 15]. It is commonly known that regular exercise is a proven and effective means to reduce weight and/or prevent the development of obesity or it's associated health risks. Even so, this is a difficult task and one that proves challenging for many individuals. Therefore, exercise is not a viable enough option for many patients that struggle to find the willpower or with conditions that keep them from being able to exercise. Following a healthy diet that restricts the intake of calories that is sustainable could be another option. However, both diet and exercise must be adopted into one's lifestyle and must be followed for a long time to appreciate any serious impact [16]. Besides surgical intervention there are no quick remedies for this disease and even this does not offer long-lasting benefits for many patients [17]. With that in mind, understanding the mechanisms that promote the onset/development of obesity are important for creating new therapies that could help patients combat the disease.

The idea of programming adult diseases like obesity *in utero* is now accepted and studies have shown that maternal exposure to factors such as stress and HFD during pregnancy developmentally program obesity in the offspring [3]. Therefore, I will focus on the role of prenatal stress programming obesity in offspring followed by HFD exposure

and how it modulates levels of neurotransmitters in specific brain regions associated with feeding regulation.

1.3 Diet

Diet-induced obesity has been an area of intense study as increased food intake has been shown to be one of the main environmental contributors to the development of obesity. HFD, high energy diet (HED), and high sugar diet (HSD), including HSD focusing specifically on fructose, have been shown to increase food consumption or cause "hyperphagia" [18-22]. Animals fed HFD have demonstrated increased adipose tissue deposition, insulin resistance, and hyperleptinemia which are all hallmarks of obesity [23]. It is believed that HFD causes hyperphagia by decreased satiation leading to overconsumption during meals. Individuals consuming HFD may have also developed physical alterations in the gastrointestinal tract allowing increased fat absorption. A previous study demonstrated that HFD may decrease satiation sensitivity by disrupting the signaling of the gut peptide, cholecystokinin (CCK) which is secreted in the small intestine and plays a role in regulating gut motility, gastric emptying, gastric acid and pancreatic enzyme secretion, and gall bladder contraction [22, 24, 25]. CCK is known to decrease meal size when administered exogenously [26], therefore a disruption in CCK signaling could lead to increased meal size resulting in higher energy consumption. Besides CCK, other factors may also be involved in affecting this complex metabolic system independently causing increased food consumption. Previous studies have shown that leptin, the adipocyte hormone, that generally suppresses feeding [27] may also be involved in this phenomenon. Leptin and CCK signaling may be reversed or blunted respectively in response to HFD [28]. Therefore, HFD may alter leptin and CCK signaling in combination causing a

disruption to this system leading to increased food consumption as has been observed in HFD-induced obesity. In addition to promoting hyperphagia, HFD causes systemic inflammation including neuroinflammation in the brain which will be discussed later. HFD also impairs cognitive performance incredibly quickly, as short as 3 days in aged adult rats [29]. This impaired cognitive performance can lead to conditions such as dementia in aged adults[30]. As mentioned previously, obesity has a direct association with increased BMI scores. There have even been comparisons linking increased BMI with decreased whole brain volume in middle aged human patients[31]. These mechanisms in which HFD affects the body can be described as increasing food consumption, increasing adipose tissue deposition, causing systemic inflammation, causing inflammation within the brain leading to cognitive deficits, and causing metabolic dysbiosis which will be described later. This highlights the incredibly negative effect of HFD on the body.

1.4 Peripheral and Central Mediators of Feeding Behavior

Peripheral signals regulate feeding behavior through hormonal and neuropeptide mediators such as: ghrelin, neuropeptide Y (NPY), leptin, and insulin which then act on the brain [32]. Peripheral mediators are located/produced in the gastrointestinal tract (GI or "gut") which is where nutrient digestion and absorption into the bloodstream occurs after eating (the humoral part of the neuro-hormonal gut-brain axis). These peripheral mediators are also perfectly positioned in the gut to act on the afferent nerve terminals of the vagus nerve (the neural part of the neuro-hormonal gut-brain axis) [33]. The vagus nerve is the major connection in the gut-brain circuitry mediating food intake and digestion. The vagal afferent nerve terminals are in the gut wall which then sends signals through the vagus nerve to the nucleus tractus solitarius (NTS) in the brainstem which

subsequently sends signals to the hypothalamus before then signaling to other higher centers and/or reward centers [33]. Ghrelin stimulates orexigenic peptides, increases food intake, and plays a role in weight gain. Leptin, one of the peptide hormones that is secreted mainly by adipose tissues, acts through the afferent signaling pathway informing the hypothalamus of the body's satiation. The levels of leptin convey the body's level of adiposity. When leptin is activated, it acts through the stimulation of anorexigenic peptides and inhibition of orexigenic peptides, inhibiting and stimulating appetite respectively. Leptin stimulates expressions of anorexigenic peptides proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) neurons, while inhibiting orexigenic peptides agouti-related protein (AGRP) neurons as well as neuropeptide Y (NPY), which is specifically secreted in the arcuate nucleus of the hypothalamus [34].

While obesity affects the body in many ways as mentioned previously, there has specifically been an area of intense investigation on the effects it has on the microbiota. While the interconnectedness of diseases and specific bacteria have long been reported, the emergence of ongoing research during the last two decades has found that microbiota positively influence human physiology with its immeasurable and diverse nature, serving multiple roles. Bifidobacterium, a gram-positive obligate anaerobe which resides in the lower part of the gastrointestinal tract, functions to reduce diarrhea, constipation, and promote gut health. The symbiotic interactions between intestinal microorganisms and their human host are thought to influence gut motility and digestion, intestinal barrier homeostasis, nutrient absorption, fat distribution, immune defense, and the activation of the vagus nerve [35, 36].

Lipopolysaccharides (LPS) are glycolipids located in the outer membrane of gram-negative bacteria, acting as endotoxins. They consist of a core lipid structure and contain polysaccharide components [37]. Consumption of a high-fat diet has been shown to disrupt the integrity of the outer membrane of gram-negative bacteria, thus promoting the release of LPS. Serving as a biomarker to metabolic disorders, increased concentrations of LPS have been shown to initiate a cascade of inflammatory events, such as secretions of proinflammatory cytokines like interleukin-6 or tumor necrosis factor, while also possibly playing a role in the inflammatory processes of insulin resistance [38]. When treating mice with LPS, increased inflammatory markers, as well as increased weight gain, were shown in adipose, muscle, and especially liver tissue [39].

Proinflammatory LPS chemicals are comprised of an O-polysaccharide chain, an R-core, and a lipid- A moiety. Whether it is commensal or pathogenic, is dependent upon which chain it contains. The O-polysaccharide chain is responsible for carrying the LPS antigenic property that targets the complementary system. Following the activation of the host complement, the characteristics and the structure of the O-polysaccharide chain is the determining factor to the degree of bacterial lysis [40]. Causing great turmoil to the gut microbiome, the dysbiosis of microbiome has the potential to elevate proinflammatory LPS chemicals. This in turn results in a myriad of complications, as inflammatory cytokines originating in the gut can affect the stimulation of afferent vagal nerves, impairing the host's connection of the gut brain axis [37].

The hypothalamus is the region of the brain that plays a major role in feeding regulation through incorporating orexigenic (stimulates hunger/appetite; suppresses satiety) signals such as ghrelin and NPY and anorexigenic signals (suppresses hunger/appetite;

stimulates satiety) such as leptin and insulin. The hypothalamus also plays a role in signaling central mediators that regulate feeding by acting on hypothalamic areas such as the arcuate nucleus and others. Central mediators include adenosine monophosphate kinase (AMPK), neuropeptide Y (NPY), agouti-related peptide (AgRP), c-Fos, proopiomelanocortin (POMC), orexin, endocannabinoids (anandamide and 2-AG), and Cb1 receptor signaling [32]. Anatomically, the hypothalamus is located ventral to the thalamus and is located rostral to the midbrain. Serving as a vital regulator of many homeostatic functions, the hypothalamus receives information signals from the peripheral organs through the vagal afferent pathway. The hypothalamus provides the linkage between the endocrine and nervous system, stimulating and inhibiting parasympathetic and sympathetic fibers. Receptors in the hypothalamus regulate and serve as the control center for food intake and appetite. It serves the purpose of controlling the body's temperature, while managing the control of stress. Its connection to the pituitary gland through the hypophyseal tract, fixated on the tubercle region of the hypothalamus, gives way to the secretion of hormones in the endocrine system. This connection allows for the process of growth, production of reproductive hormones, and maintenance of the body's metabolism [41].

Eleven major nuclei make up the hypothalamus and are based on the zones in which they reside, each with different functional roles [42]. The nuclei we are interested in, as they play a role in feeding, are the paraventricular nucleus (PVN), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), arcuate nucleus (AN), and the lateral hypothalamic area (LHA). Two specific nuclei that are of paramount importance to the periventricular zone regarding food intake are the AN and the PVN. The AN has been shown to play a dynamic factor in energy homeostasis, food-intake, satiety, thirst, and

circadian rhythms. The AN contains POMC or AGRP/NPY, both composed of leptin receptors for the hormone to induce expression. When consuming HFD with large amounts of long chain saturated FAs, hypothalamic inflammation is induced, ultimately resulting in the disruption of leptin signaling [42]. Impairment to the leptin signaling pathway has been shown to result in obesity and validifies the importance of leptin's role in mediating energy balances [43]. The PVN plays a prominent role in neuroendocrine and autonomic nervous system regulation, displaying roles and providing maintenance to the hypothalamus-pituitary axis, the thyroid axis, the reproductive axis, growth and development, the regulation of body fluid balance, along with the assistance of the functions of the gastrointestinal tract and the cardiovascular system [44]. The PVN was found to be a primary site in the mediation of adrenergic stimulation, when injecting mice with norepinephrine, on feeding and drinking. The results for a study dated back to 1978 identified PVN as one of, if not, the most important regulators for food intake and obesity. Isolating regions of each nucleus in the hypothalamus, the study found that the PVN happened to be the only nuclei to elicit the response to the injections of norepinephrine, thus conveying its prominent role in food regulation [44, 45].

The DMN plays a role in the motor control of food anticipation. This mechanism will cause animals to exhibit increased motor food-anticipatory activity or FAA a few hours before a meal when kept on a normal daily food schedule. A lesion to the DMN will disrupt this mechanism and decrease FAA similarly to animals that have undergone food restriction [46]. The DMN also connects to other brain regions such as the AN which plays a major role in feeding regulation as previously mentioned [47]. The DMN contains neurons that express leptin receptor which a recent study has shown that chronic

silencing of these neurons caused hyperphagia that lasted for multiple days leading to increased body weight and body fat accumulation, serum leptin, fasting blood glucose, and plasma insulin. These are all factors of obesity and insulin resistance as previously mentioned and therefore indicates the major role of DMN neurons on feeding regulation and energy homeostasis [48].

Stimulation to other nuclei in the hypothalamus such as the LHA neurons has been shown to produce eating in sated animals and active firing of dopamine neurons as well. Chemical lesions or impairments of neuronal pathways of dopamine results in severe aphagia, which is problematic as dopamine comprises a major connection to the control of feeding [49]. Additional nuclei in the medial region, such as the VMN, regulate feeding behavior. Disruption to the VMN has been shown to result in starvation [50]. The VMN, commonly known as the brain's "satiety center," has historically been an area of interest that researchers have expressed regarding its effects on feeding behavior and obesity. A series of studies that were conducted by the researchers Hetherington and Ranson in the early 1940s shed light on the establishment that lesions to the VMN resulted in excessive levels of food intake and adiposity [51]. Even though studies have shown animals with ventromedial hypothalamus lesions to become obese, the outcome is not in part due to damage to the neuronal satiety center itself. Previous hypothesis has purported that ventromedial hypothalamus lesions could result in the dampening of "cephalic reflexes" regarding food stimuli. Affecting the pathways of the sympathetic nervous system, reflexes such as pancreatic, salivary, and gastric secretions, ultimately become diminished, which amplifies and encourages a positive feedback mechanism.

1.5 Immune System and Inflammation

The innate immune system is the body's first line of defense against foreign substances. It reacts to these foreign substances through a term known as “nonspecific” and/or “general" immune system. The innate immune system consists of protection by mucous membrane and skin and protection by immune system cells and proteins. In contrast, the adaptive immune system goes into action when the innate immune system is unable to destroy the foreign substance. It targets the specific pathogen by identifying the pathogen to launch a slower but more accurate response. When the skin becomes infected by foreign substances, the innate immune system activates cells at or call cells to the area of infection, which results in inflammation.

Macrophages, effector cells of the innate immune system, have a defense function against pathogens and maintaining homeostasis of the body. Macrophages use a variety of methods to phagocytize and kill pathogens, each in a different way. When macrophages are overwhelmed with pathogens, they employ an inflammatory response. Microglial cells are the resident macrophage population in the CNS and play a major role in the brain’s innate immune system. Microglial cells have proinflammatory effector functions following their activation. Microglial cells are responsible for early control of infections and recruitment of other cells from the adaptive immune system to neutralize pathogens [52, 53].

When microglia become activated, neuroinflammation occurs to cope against certain inflammatory challenges. Neuroinflammation is the activation of the CNS’s innate immune system response to an inflammatory challenge. The characterization of this inflammation is the cellular and molecular changes experienced in the CNS.

Neuroinflammation is caused by a variety of cues, such as, infection, brain trauma, toxins, and autoimmunity. In the CNS, microglia are activated due to the response of the cues previously mentioned [54]. On the other hand, peripheral inflammation is the activation of the innate or adaptive immune system. This inflammation is caused by the release of proinflammatory cytokine to attempt to combat pathological stimuli apart from the CNS. Cues for inflammation of periphery is similar, but not limited to those of neuroinflammation in respect to the location of the pathological stimuli. The blood-brain barrier (BBB), located between the CNS tissues and peripheral circulation, regulates cellular exchange between blood vessels and brain parenchyma [55].

High-fat diets (HFD) can also cause neuroinflammation in the CNS. Inflammation caused in the CNS, as mentioned before, is due to microglia activation. Microglia play a major role in the immune system as the reason for inflammation. HFD can trigger the activation of microglia and inflammation in the hypothalamus before causing changes in body weight [56]. HFD induces an increase in mitochondrial uncoupling protein 2 (Ucp2) mRNA expression in the hypothalamic microglia. The increase in Ucp2 leads to an increase in HFD-induced inflammation, obesity, and POMC synaptic plasticity. The changes in the microglia mitochondrial mechanism are the major component in the microglia activation [57, 58]. HFD can not only affect the activation of microglial cells, but also affect cytokine release. SFAs have been linked to the activation of TLR4 receptors in the hypothalamic region and causes the stimulation of cytokines release. Cytokines are substances (such as, interferon and growth factors) that influence other cells when released by cells in the immune system. When microglial cells are activated, they release pro-inflammatory cytokines (such as, IL-1, IL-6, and TNF-α) which are intended to

prevent damage to tissue in the CNS but may be toxic to neurons and glial cells. This indicates that the possible implication of HFD leads to brain inflammation and progression of neurodegenerative disorders (specifically in the hypothalamus) due to over stimulation of cytokine release [59-61].

Neuronal inflammation has its positive effects, as mentioned previously, in providing support in infection, brain trauma, toxins, and autoimmunity, but excess neuroinflammation can be detrimental as well. HFD induces excess neuronal inflammation which can lead to problems such as diminishing cognitive abilities, decline in memory and memory disorders (Alzheimer's Disease and dementia), neurological disorders (Parkinson's Disease), and neuropsychiatric disorders (depression and anxiety) [62-66]. The introduction of anti-inflammatory drugs leads to decrease in neuronal inflammation to help alleviate problems such as the ones mentioned. Anti-inflammatory drugs are drugs that are used to reduce pain, reduce inflammation, and bring down high temperature or fever. They can be used to ease the pain of many conditions, such as, muscle pain, cold, flu, and headaches. High levels of macrophages can cause pain, inflammation, and temperature increase. Anti-inflammatory drugs help reduce these symptoms by blocking the production of macrophages. One effect of anti-inflammatory drugs is altering the level of macrophages in the brain, the main one being microglia. When there is a demand for macrophages in peripheral tissue, monocytes reform into macrophages [67]. The cytokine colony-stimulating factor 1 receptor (CSF1R) controls the differentiation, polarization, and transmigration of macrophages which is controlled by CSF1 levels in the environment [68]. The increased levels of CSF1 circulating in genetically obese mice are treated with a CSFR1R neutralizing monoclonal antibody.

1.6 Pexidartinib (PLX)

Pexidartinib (PLX 3397) is an oral tyrosine kinase inhibitor of the CSF1R that reduces macrophage levels [69]. PLX3397 targets the CSF1/CSF1R pathway by being an inhibitor of the CSF1R by stimulating the autoinhibited state of the CSF1R. The CSF1R is inhibited by the interaction of PLX3397 with the juxtamembrane of the CSF1R which plays a role in folding and inactivation of the kinase domain and preventing the binding of CSF1 and ATP to the juxtamembrane region. Since the CSF1/CSF1R pathway is being inhibited, PLX3397 downregulates the proliferation and downmodulates macrophages [70]. For the purposes of this research, the effects of PLX3397 anti-inflammatory drug on microglia in the CNS were investigated. Microglial cells are a major component of brain inflammation and neurodegeneration. This causes microglial cells to be a major target for anti-inflammatory drugs within the brain [52]. PLX3397 induces a strong decrease in microglial proliferation without affecting other cell types in the CNS [53]. PLX3397 has other therapeutic effects aside from causing anti-inflammatory responses by reducing microglial activation in the CNS. In a recent study, the introduction of PLX3397 has proven to improve chronic demyelination in neurodegenerative diseases, which is connected to the possible adjustment of milieu that surrounds the axons to retain remyelination. When rats are administered cuprizone, it causes demyelination of fibers, destruction of nerve fibers, and formation of gaps between layers of myelin sheaths in the corpus callosum, which are counteracted with PLX3397 as therapy to improve remyelination process [71]. PLX3397 treatment promotes the reduction of macrophages present in the adipose tissue, which in turn reduces inflammation and pain in the targeted areas. Macrophage accumulation in adipose tissue has been associated with the mechanism driving obesity-related

metabolic dysfunction and the cytokine CSF1 stimulation in macrophage differentiation and migration stages. PLX3397, being an inhibitor of CSF1R, has been proven to significantly reduce adipose tissue macrophage levels of mice fed with chow or HFD [69]. PLX3397 treatment provides a blockade for the CSF1R which improves the success of adoptive T-cell therapy (ACT) immunotherapy. PLX3397 is successful in ACT immunotherapy by inhibiting intratumoral accumulation of immunosuppressive macrophages [72].

1.7 Prenatal Stress Programming and the HPA Axis

The idea of "programming" was developed by David Barker in 1995 who conducted epidemiological studies in which he observed that mothers who were exposed to a natural disaster such as a famine and were subjected to severe undernutrition during pregnancy gave birth to underweight babies. These babies developed coronary artery disease when they became adults [73, 74]. This later became the 'Barker hypothesis' or "the developmental origins of health and disease (DOHAD)" concept. This maternal or 'prenatal programming' also primed the offspring to develop obesity and metabolic syndrome following the challenges previously mentioned [75]. *In utero,* during the period of poor nutrition, it is thought that the fetus undergoes adaptations at the tissue level to survive. These maladaptive changes cause the offspring to develop obesity and metabolic syndrome responses in the postnatal period. These prenatal adaptations cause the body to convert any available energy into fat for survival. This mechanism, called the 'thrifty genotype or thrifty phenotype hypothesis' goes against what would be expected in a normal animal or individual such that when there is postnatal over feeding, the prenatal

adaptations that were born out of necessity for the fetus to survive end up working against itself and causes obesity [76].

Now years later after the Barker's hypothesis has been further investigated, multiple mechanisms have been suggested to be involved in this process of prenatal programming *in utero*. Of specific interest is the idea that prenatal stress and undernutrition may cause an effect on the hypothalamo-pituitary adrenal (HPA) axis whereby it becomes hyperactivated and the offspring in the postnatal period become hypersensitive to stress. This causes increases in the expression of hypothalamic corticotropin releasing hormone (CRH), circulating adrenocorticotropin (ACTH), circulating glucocorticoids, and hippocampal 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1) expression. Moreover, the offspring have also been shown to be insulin resistant. It has then been suggested that all these changes then culminate in the development of obesity and associated disorders such as type 2 diabetes and other behavioral disorders [5, 77-81]. This phenomenon by which prenatal stress programs the metabolism of the offspring to the development of obesity is not fully understood.

The HPA axis mentioned previously, also known as the 'stress axis,' was first identified to cause physiological responses in 1939 by Hans Selye when he discovered that there was a connection between stress and the adrenal gland [82]. Today, many studies and decades later, we know that the HPA axis is composed of CRH neurons located in the hypothalamus, corticotrophs in the anterior pituitary gland and the adrenal cortex. The HPA axis works by a cascade of hormones which is initiated by stressful stimuli triggering noradrenergic neurons in the brain stem regions such as the NTS, rostral ventrolateral medulla (RVLM or A1) and the locus coeruleus (LC or A6) which leads to stimulation of

CRH neurons in the PVN by the release of norepinephrine (NE). CRH then stimulates the release of adrenocorticotropin (ACTH) from corticotrophs in the anterior pituitary which then enters the systemic circulation to act on the adrenal gland to stimulate corticosterone secretion in rats/mice (cortisol in humans). To ensure that this cascade ends when there are no more stressful stimuli, a negative feedback loop exists. This is achieved by the binding of glucocorticoids to their receptors, glucocorticoid receptors (GR), located in the brain stem noradrenergic neurons, the CRH neurons in the PVN, and the anterior pituitary [83-86].

During stressful events, the HPA axis can be hyperactivated and increase the amount of circulating corticosterone levels that helps to mobilize fat and glycogen stores for rapid energy release to deal with a stressful situation. This is called the "fight or flight" response [87]. Frequent acute and/or chronic stress exposure could potentially lead to excess circulating glucocorticoids for long periods of time and may lead to the development of maladaptive responses that affect metabolic function. The idea of stress promoting obesity could be explained by the fact that GR are highly expressed in visceral fat [88] and are therefore more responsive to circulating glucocorticoids. The binding of glucocorticoids to GR activates an enzyme called lipoprotein lipase (LPL). LPL then causes increased energy storage and visceral fat accumulation and as previously mentioned, increased visceral fat accumulation promotes a pro-inflammatory state which sustains HPA activation. Therefore, this mechanism of stress activating the HPA axis followed by fat accumulation and a pro-inflammatory state sustaining HPA activity could pose a problem as they form a loop that could be hard to break [89-92].

There are several lines of evidence that support a role for glucocorticoids in obesity. Cushing's syndrome that is characterized by higher circulating levels of glucocorticoids is marked by increased food intake, reduced lean mass, increase in visceral fat deposition and truncal obesity [93]. Moreover, patients with central obesity have higher levels of fasting cortisol [94], and a loss of diurnal variation [95]. Cortisol levels have also been found to correlate with waist circumference [96]. Obese individuals also excrete more cortisol through the urine [97].

1.8 Monoamine Neurotransmitters

The three monoamine neurotransmitters are norepinephrine/noradrenaline (NE), dopamine (DA), and serotonin (5HT). These neurotransmitters play a significant role in the body by interacting with hormones in the brain for multiple physiological functions. NE is made in the brain stem and projects to different regions of the brain. As mentioned previously, NE and the PVN are major components of the HPA or stress axis. A study showed that when NE is injected into the PVN, hyperphagia results [98]. Leptin may play a role in inhibiting NE release in the PVN, but one of the hallmarks of obesity is hyperleptinemia, therefore there may be resistance to the leptin in the brain allowing NE to be released uninhibited [99, 100].

DA is produced mostly in the substantia nigra and the ventral tegmental area (VTA) and is a major component of the reward system. DA and the reward system are what motivates individuals to consume highly palatable foods, even when hunger is absent. The exact mechanism of DA is not fully understood as it can both stimulate and inhibit feeding depending on the region of the brain and its interaction with other hormones such as leptin [101-104]. In addition to the reward system, DA plays a role in the

homeostatic hypothalamic system which, as the name suggests, controls energy homeostasis. DA neurons are present throughout the hypothalamus and, as previously mentioned, seem to play a major role of suppressing food intake in the AN and the LHA while stimulating food intake in the VMH. DA neurons project to many of the hypothalamic nuclei allowing DA to act on feeding regulation independently and with other hypothalamic neurons such as AgRP/NPY and POMC to regulate energy homeostasis [104, 105].

Cases of disorders such as anxiety and depression seem to be constantly on the rise and the role 5HT on mood have led to the development of drugs that help increase the absorption of 5HT as it is the "feel good" hormone and helps treat the symptoms from such disorders. Examples of such drugs are selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs) which block the reabsorption of serotonin and norepinephrine to the nerve terminals from which they were released, increasing their concentration in the synaptic cleft [106]. In addition to 5HT's effect on mood, it has also been shown in humans and animals to decrease food consumption and weight and increase energy expenditure due to the action of serotoninergic receptors in the hypothalamus. A feedback system has been observed in that when carbohydrates are consumed, 5HT is produced which then inhibits further consumption of carbohydrate dense meals [107]. This effect by 5HT and the other effects by the other monoamine neurotransmitters highlight the dynamic role they play in feeding regulation and energy homeostasis.

1.9 DIO/DR Animal Model

As mentioned previously, the development of obesity is commonly caused by environmental factors, but genetics also plays a role in combination with these factors. This multifactorial cause of obesity, environmental and genetic, is the most common, but studies have shown that there are two other genetic mechanisms that produce specific obesity phenotypes. These other, less common genetic mechanisms are syndromic and monogenic obesity. Syndromic obesity is associated with certain clinical disorders while monogenic obesity is due to a single gene mutation which can be inherited [12]. Genetics has also been shown to play a role as individuals consuming western diets can remain relatively slim while others gain weight and have difficulty in reducing body weight/fat accumulation. This indicates the genetic role of obesity resistance vs susceptibility [108]. The multifactorial cause of obesity is not fully understood due to the complex nature of the onset of the disease. Therefore, to study the development of obesity using animal models, a DIO and DR polygenic rat model was developed from Sprague-Dawley rats as these rats respond to HFD similarly to humans. The DIO-DR rat model was developed by feeding adult male and female rats high in energy diets and identifying the animals that increased in body weight vs those that decreased. These animals in the upper and lower quartile of body weight were then mated according to their phenotype and this was continued to 20+ generations. This process therefore classified the phenotype of these animals as being obesity prone or obesity resistant. Now, this model has been used for many studies including HFD studies as the rats respond similarly humans. This model is desirable for a programming study that involves stress and HFD exposure as the gestational

period for rats is much shorter than humans (21 days), but the way the animals respond to stress and HFD should be similar to humans [109].

1.10 The use of Surgical stress as a prenatal stress paradigm

Maternal surgery has been used as a prenatal stress paradigm in a few animal models [110, 111]. In the studies described in this dissertation, pregnant DIO and DR dams were subjected to jugular catheterization to measure changes in circulating parameters during pregnancy. The purpose was to investigate differences in specific hormones such as adiponectin and leptin and changes in cytokines such as IL-1beta, IL-6 and TNF alpha that could potentially influence the development of obesity in the offspring. The hypothesis was that differences in these 5 parameters during pregnancy were contributing to the phenotype differences and the propensity of the rats to become obese or not in adult life. During the course of these studies, it was found that offspring from animals that were subjected to jugular catheterization during each week of pregnancy, a surgery that lasted for a maximum of 10 minutes, resulted in the offspring consuming more food from weaning to adulthood compared to offspring born from un-catheterized dams. This paradigm was termed as "surgical prenatal stress" and formed the basis for the studies described in chapters 2 and 3.

1.11 Overall Objective

The overall objective of my dissertation is to understand the mechanisms involved in promoting obesity. While the first chapter investigates the role of inflammation, chapters 2 and 3 study the effects of prenatal surgical stress on the development of obesity. HFD was used as a common environmental factor to initiate the inflammatory or the stress response, since it is frequently associated with propitiation of obesity. The studies

described in the following chapters were designed to test the following hypotheses: 1) The anti-inflammatory drug, PLX3397 will reduce microglia activation (neuroinflammation) in brain nuclei involved in regulating metabolic functions in rats fed HFD; 2) Maternal surgical stress will increase food intake and body weight of DIO and DR offspring fed HFD and 3) Prenatal surgical stress will increase levels of monoamine neurotransmitter concentrations in nuclei involved in regulating feeding in offspring fed HFD. These studies will help to determine if the anti-inflammatory drug PLX3397 can be a useful therapeutic intervention for obesity. We will also understand if changes in hormone levels and feeding circuits can contribute to obesity in offspring subjected to prenatal stress.

CHAPTER 2

THE ROLE OF PLX3397 AFTER SHORT-TERM CONSUMPTION OF HIGH FAT DIET ON MICROGLIA ACTIVATION IN THE HYPOTHALAMUS OF MALE AND FEMALE SPRAGUE-DAWLEY RATS

Brent P. Norwood, Flynn P. O'Connell, Krzysztof Czaja, Andras Hajnal, and Patricia M. Di Lorenzo. University of Georgia portion of manuscript submitted to *Neuroglia*, 10/27/22.

2.1 ABSTRACT

Our lab has previously shown that HFD causes an increase in food consumption and inflammation in the CNS [19, 56]. Therefore, HFD promotes the development of obesity, a major healthcare concern in the US and around the world. Microglial cells are activated in the CNS upon short-term (4 weeks) consumption of HFD and are an indicator of inflammation. The aim of this study was to investigate the short term (6 weeks) use of an anti-inflammatory drug, Pexidartinib (PLX3397), to reduce the activation of microglial cells in the hypothalamus. Male and female Sprague-Dawley rats were fed either CD or HFD for 10 weeks and then treated with either CD, HFD, CD+PLX, or HFD+PLX for 6 weeks. The rats were sacrificed at the end of the 10 weeks and brains were collected to be sectioned and stained using Iba-1 for microglia activation quantification. Microglia activation was increased in specific nuclei of the hypothalamus that are related to feeding regulation after consumption of HFD. PLX3397 significantly decreased or prevented microglial cell activation in the hypothalamic nuclei related to feeding regulation. These data suggest that anti-inflammatory drugs such as PLX3397 may be a viable treatment option for patients with obesity in combination with other classical methods such as diet and exercise.

2.2 INTRODUCTION

The World Health Organization (WHO) has defined obesity as a worldwide epidemic for the past two decades, and in 2021, about 38.9% of the world's population is estimated to be obese (John Elflein, Obesity worldwide- Statistics and facts. Statista 2022) [112, 113]. According to the Center for Disease Control and Prevention (CDC), the obesity rate in the US increased from 30.5% - 41.9% of Americans from 1999-2000 to 2021 respectively. This is an alarming rate as obesity related conditions such as heart disease, stroke, Type-2 diabetes, and cancer are leading [112]causes of death in the US for preventable diseases [13]. The prevalence of obesity and its related conditions cause concern for the foreseeable future, as its etiology is not entirely understood.

High fat diet (HFD) leads to pathophysiological changes in the gut microbiome and parts of the hypothalamus. This connection can be better visualized in the gut-brain axis (figure 2.1). The vagus nerve is the bidirectional communication pathway for the regulation of homeostasis throughout the visceral organs. Vagal afferent neurons transmit visceral sensory information to the hypothalamus [114], signaling hunger and satiation through the secretion of ghrelin and leptin [34]. Consumption of HFD has been shown to promote the release of lipopolysaccharide (LPS) in the gut. Increased concentrations of LPS have been shown to initiate a cascade of inflammatory events, such as secretions of proinflammatory cytokines, while also possibly playing a role in the inflammatory processes of insulin resistance [38].

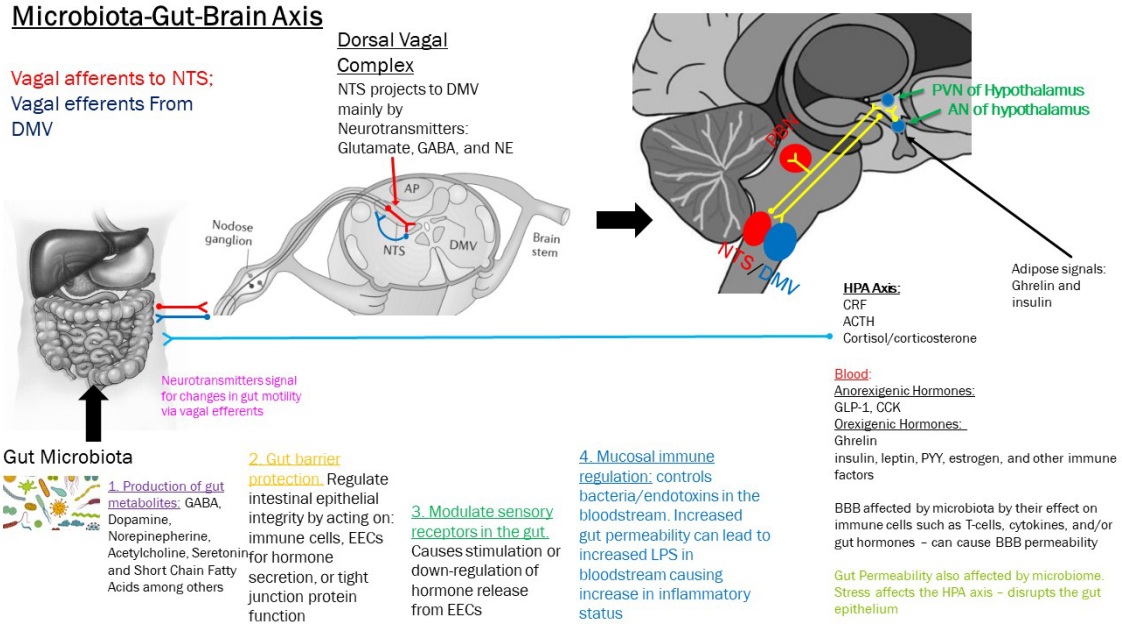

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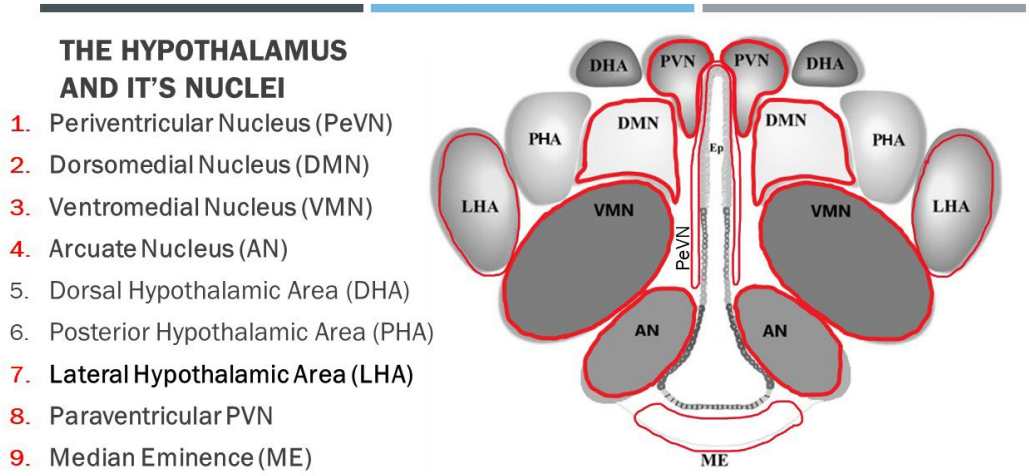
Figure 2.1: Microbiota-Gut-Brain Axis.

The figure above depicts the gut, brain vagal bidirectional communication pathway. They communicate via vagal afferent neurons (red) from the gut to the nucleus of the solitary tract (NTS), Parabrachial nucleus (PBN), and hypothalamus. Then they communicate from the brain to the gut via vagal efferents neurons (blue) from the hypothalamus, dorsal motor nucleus of the vagus (DMV), and HPA axis. The roles of the gut microbiota are: 1. Production of gut metabolites, 2. Gut barrier protection, 3. Modulate sensory receptors in the gut, and 4. Mucosal immune regulation.

The proliferation of glial cells promoted from the inflammatory pathways, such as toll-like receptors (TLRs) and canonical cytokines, have thus far conveyed a correlative linkage to the impairment of the hypothalamus. This impairment can further disrupt the

regulation of feeding behaviors which plays a prominent role in obesity [116]. Microglial cells are the resident macrophage population in the CNS. Microglia have a proinflammatory effector function, neuroinflammation, following their activation [52, 53] which is the central nervous system's (CNS) innate immune response to an inflammatory challenge. Neuroinflammation is caused by a variety of cues, such as, infection, brain trauma, toxins, and autoimmunity. Microglia are therefore activated due to the response of these cues [54]. Therefore, HFD can affect the activation of microglial cells, but also affect cytokine release. These pro-inflammatory cytokines (such as, IL-1, IL-6, and TNF-α) are intended to prevent damage to tissue in the CNS but may be toxic to neurons and glial cells. Short chain fatty acids (SFAs) have been linked to the activation of TLR4 receptors in the hypothalamic region and causes the stimulation of cytokines release as well. This indicates the possible implication of HFD causing brain inflammation and the progression of neurodegenerative disorders (specifically in the hypothalamus) due to over stimulation of cytokine release [59-61].

The hypothalamus serves as a vital regulator of many homeostatic functions and provides the linkage between the endocrine and nervous system. Receptors in the hypothalamus regulate and serve as the control center for food intake and appetite. Its connection to the pituitary gland gives way to the secretion of hormones in the endocrine system [41]. The hypothalamus is composed of many nuclei but, the nuclei we are interested in, as they play a role in feeding, are the paraventricular nucleus (PVN), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), arcuate nucleus (AN), and the lateral hypothalamic area (LHA) (figure 2.2). Two specific nuclei that are of paramount importance regarding food intake are the AN and the PVN [42].



[117]

Figure 2.2: The hypothalamic nuclei involved in feeding regulation.

The figure represents a coronal cross section of the hypothalamus with the nuclei of interest involved in feeding regulation highlighted in red: 1. PeVN, 2. DMN, 3. VMN, 4. AN, 7. LHA, 8. PVN, 9. ME.

The DMN, VMN, and AN are all in the same region and play a dynamic factor in energy homeostasis, food-intake, satiety, thirst, and circadian rhythms. The PVN plays a prominent role in neuroendocrine and autonomic nervous system regulation, providing maintenance to the hypothalamus-pituitary axis among others [44]. The PVN is a primary site in the mediation of adrenergic stimulation on feeding and drinking by responding to norepinephrine, thus making the PVN one of, if not the most, important regulators for

food intake and obesity [44, 45]. Stimulation to other nuclei in the hypothalamus such as the lateral hypothalamic area (LHA) neurons has been shown to promote eating in sated animals. The VMN, commonly known as the brain's "satiety center," has been an area of interest as lesions have been shown to result in excessive levels of food intake and adiposity [51]. Though studies have shown animals with VMN lesions to become obese, the mechanism by which this occurs has yet to be fully established [118]. Disruption to the ventromedial nucleus (VMN) has also been shown to result in starvation [50]. After a meal, α-melanocyte-stimulating hormone (α-MSH) binds to receptors in the PVN, DMN, VMN, and LHA causing an anorexigenic effect [42].

HFD induces excess neuronal inflammation which can lead to cognitive, neurological, and/or neuropsychiatric disorders [62-66]. Anti-inflammatory drugs can decrease neuronal inflammation to help alleviate problems such as these. High levels of macrophages can cause pain, inflammation, and temperature increase. Anti-inflammatory drugs help reduce these symptoms by blocking the production of macrophages. One effect of anti-inflammatory drugs is altering the level of macrophages in the brain: microglia [67]. The cytokine colony-stimulating factor 1 receptor (CSF1R) controls the differentiation, polarization, and transmigration of macrophages which is controlled by CSF1 levels in the environment [68]. The increased levels of CSF1 circulating in genetically obese mice are treated with a CSFR1R neutralizing monoclonal antibody.

Pexidartinib (PLX 3397) is an oral tyrosine kinase inhibitor of the CSF1R that reduces macrophage levels [69]. PLX3397 targets the CSF1/CSF1R pathway by being an inhibitor of CSF1R by stimulating the autoinhibited state of CSF1R. Since the CSF1/CSF1R pathway is being inhibited, PLX3397 downregulates the proliferation and

downmodulates macrophages [70]. This causes microglial cells to be a major target for anti-inflammatory drugs within the brain [52]. PLX3397 induces a strong decrease in microglial proliferation without affecting other cell types in the CNS [53]. For the purposes of this research, the effects of PLX3397 anti-inflammatory drug on microglia in the CNS were investigated. Therefore, we hypothesize that anti-inflammatory drug PLX3397 will cause a decrease in microglia activation (neuroinflammation) in brain nuclei involved in metabolism regulation in male and female rats fed HFD.

2.3 MATERIALS AND METHODS

Animal Care and Dietary Manipulations

Institutional Animal Care and Use Committee of Binghamton University approved the procedures performed which were in accordance with the National Institutes of Health Animal Welfare Guide. 30 male and 27 female Sprague-Dawley rats (8 weeks old) were received from Taconic Labs, Inc. (Germantown, NY, United States). The acclimation period for the rats allowed them to get adjusted to the temperature-controlled vivarium for 7 days with a 12 h light/dark cycle (lights off at 0900 h). During acclimation, rats had ad libitum access to a standard chow diet (CD) (Purina Lab Diet 5L0D, catalog 0050795, Lab Diet, St. Louis, MO, United States; 14% fat, 29% protein, 57% carbohydrate) and water. Following the acclimation period, rats were weight-matched, single-housed and separated by their respective dietary groups. For 10 weeks, rats were given *ad libitum* access to water and either HFD (Research Diets, catalog D12451; 45% fat, 20% protein, and 35% carbohydrate) or CD. Measurements for body weight and cage food intake were taken daily.

Pexidartinib (PLX3397)

After 10 weeks of HFD or CD exposure, the pre-anti-inflammatory treatment period, rats were given access to CD, HFD, or nutritionally equivalent diets infused with the anti-inflammatory drug, Pexidartinib (PLX3397, MedKoo Biosciences, Morrisville, NC, United States), for the anti-inflammatory treatment (figure 2.3 and 2.4**)**. Pexidartinib was obtained and sent to Research Diets (New Brunswick, NJ, United States) for the infusion of the drug into the diets. Diets used were as follows: HFD (Research Diets, catalog D12451), HFD+PLX (Research Diets, 300 mg PLX/kg HFD), CD+PLX (Research Diets, 189.5 mg PLX/kg 5L0D chow diet) or CD. CD+PLX dosages were administered as the same quantity of PLX3397/kCal/gram body weight as the HFD+PLX using previously obtained food-intake data from rats consuming 5L0D chow (Weiss et al., 2018).

Experimental design

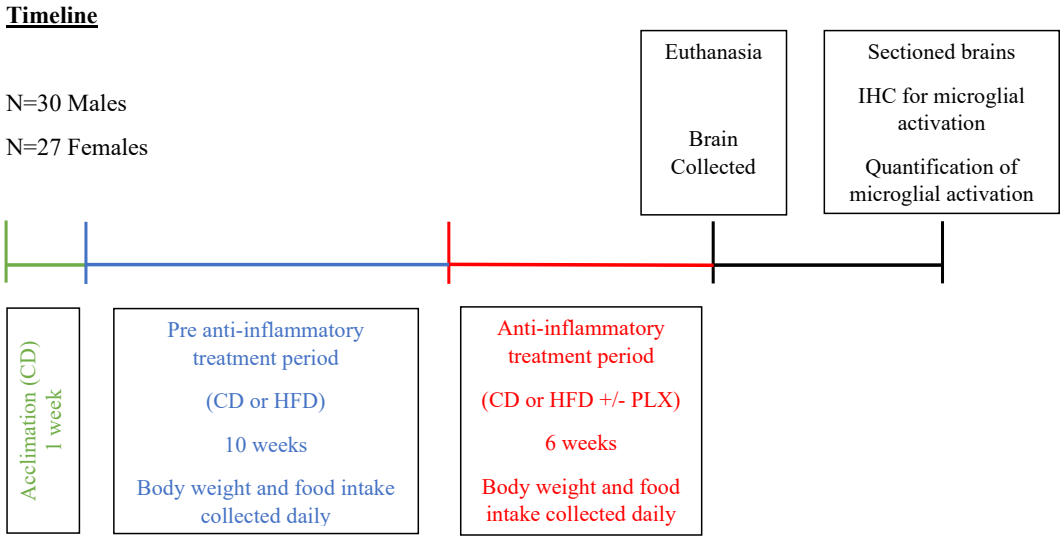


Figure 2.3: Experimental Design Timeline.

Male and Female (N=30, N=27 respectively) rats were acclimated for 1 week on standard chow diet (CD). Rats were divided into high fat diet ("HFD"; 45% fat) or CD for 10

weeks. Next, the rats were given CD, HFD, or nutritionally equal diets infused with Pexidartinib (PLX-3397; "PLX") for 6 weeks. Body weight and food intake were collected daily for the duration of the experiment. At the end of the experiment, the rats were euthanized, and the brains were collected. The hypothalamus was cryosectioned at 20 µm and 6 sections were mounted on slides. Slides were stained for microglia activation using Iba-1 antibody. The sections were imaged, and the binary area fraction of Iba-1 was measured for hypothalamic nuclei. The binary area fraction data was analyzed by one-way ANOVA and graphed.

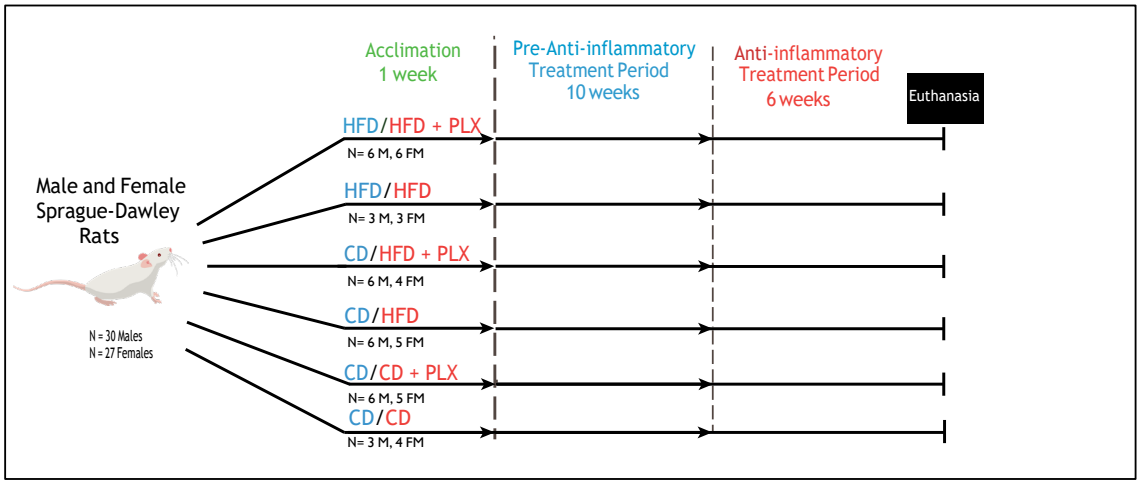


Figure 2.4: Experimental Design.

Male and Female (N=30, N=27 respectively) rats were acclimated for 1 week on standard chow diet (CD). Rats were divided into high fat diet ("HFD"; 45% fat) or CD for 10 weeks. Next, the rats were given CD, HFD, or nutritionally equal diets infused with Pexidartinib (PLX-3397; "PLX") for 6 weeks. Body weight and food intake were collected daily for the duration of the experiment. At the end of the experiment, the rats were

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Tissue Collection

The donor rats were euthanized after the anti-inflammatory treatment period. Rats were first anesthetized with CO_2. Then, they were transcardially perfused with 0.1 M phosphate-buffered saline (PBS; pH 7.4) and 4% paraformaldehyde. Brains were harvested and fixed in 4% paraformaldehyde for 2 hours. They were then soaked in a solution of 30% sucrose, 0.1% NaN_3 (Sigma-Aldrich, pH 7.4) in PBS and stored at 4°C until processed.

Sectioning

The hypothalamus was isolated from the rest of the brain by a coronal cut rostral to the cerebellum and at the level of the optic chiasm. The remaining caudal and rostral portions of the brain were stored at 4°C for future studies. The hypothalamus was placed in a specimen boat with the rostral end down to preserve the orientation of the tissue. Mounting media was applied to the hypothalamus and it was completely covered for at least 30 minutes before sectioning. The brains were cryosectioned (Leica CM1950, Leica Biosystems, Wetzlar, Germany) at 20 μm from the caudal to rostral area of the hypothalamus (between bregma −3.24 and −1.92 mm) and mounted on slides. Every slide contained 6 total sections, two rows of three sections each.

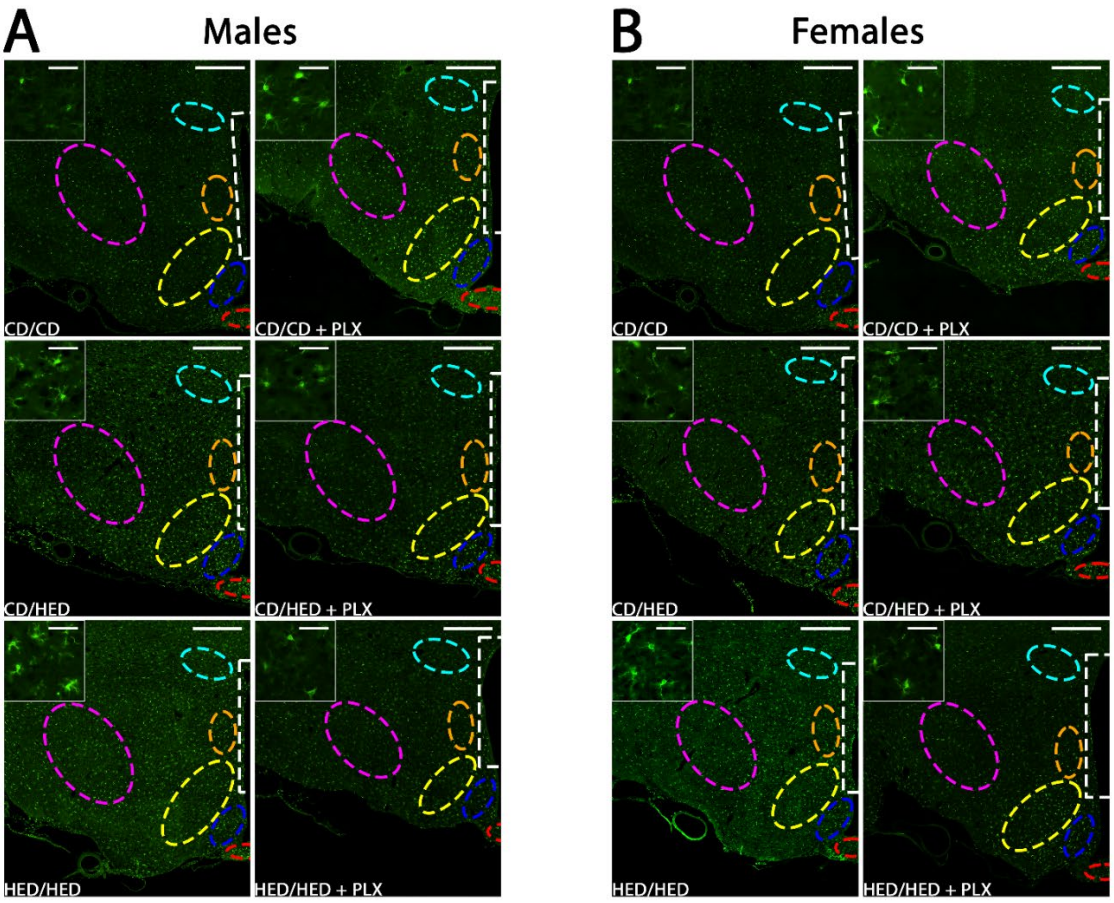
Immunohistochemistry

After the hypothalamus was cryosectioned, standard immunofluorescence was utilized to assess the microglia activation in the hypothalamus. The slides were examined under the microscope for quality assurance. Each tissue section was identified and circled with a hydrophobic pen. The tissue was rinsed three times for 10 minutes each in TPBS with mild shaking. The tissue was then permeabilized with Sodium Borohydride (1 pinch of sodium borohydride to 50 mL of PBS) for 5 minutes with mild shaking. The tissue was rinsed two additional times for five minutes each with mild shaking. 20uL of 1:1000 dilution of primary antibody (Anti-IBA-1 Rabbit in IHC-Tek antibody diluent) was applied to the tissue and incubated overnight in a humidity chamber. The following day, the tissue was rinsed three times for 10 minutes in TPBS with mild shaking. The sections were cover slipped with ProLong Gold liquid mountant without DAPI. The sections were incubated overnight with a primary antibody against ionized calcium binding adaptor molecule 1 (Iba-1, Wako Cat#019-19741, RRDI: AB_839504) followed by Alexa-488 secondary antibody (Alexa 488 Donkey anti-Rabbit, Invitrogen by Thermo Fisher Scientific, cat#A21206) for 2-hr to visualize microglia activation as previously described [119].

Imaging

Images of the hypothalamus were captured under 20× magnification with a Nikon 80i imaging photomicroscope (Nikon, Tokyo, Japan) equipped with a digital camera (Nikon Digital Sight DS-Qi1Mc), and appropriate filters for DAPI, Alexa 488, and ExtrAvidin-CY3. Images were analyzed with the Nikon Elements AR 3.0 Imaging software (Nikon, Tokyo, Japan). An average auto-exposure of 339ms was established which ensured that each immunofluorescent image of the microglia presented a consistent

brightness. For each image, the boundaries of the hypothalamus were set. The scan large image function was used to stitch together all the 20x magnification images. The hypothalamus was quantified by measuring the binary area fraction of Iba-1: the activated microglia in each region of interest (ROI). The ROI were: 1. Median Eminence, 2. Left Arcuate nucleus, 3. Right Arcuate nucleus, 4. Left Ventromedial nucleus, 5. Right Ventromedial nucleus, 6. Left Dorsomedial nucleus, 7. Right Dorsomedial nucleus, 8. Periventricular nucleus, 9. Left Periventricular nucleus, 10. Right Periventricular nucleus, 11. Left Lateral hypothalamic area, 12. Right Lateral hypothalamic area. These data were utilized to make the graphs in the results **(**Figure 2.5**)**.


A
Males
B
Females
CD/CD
CD/CD + PLX
CD/HED
CD/HED + PLX
HED/HED
HED/HED + PLX

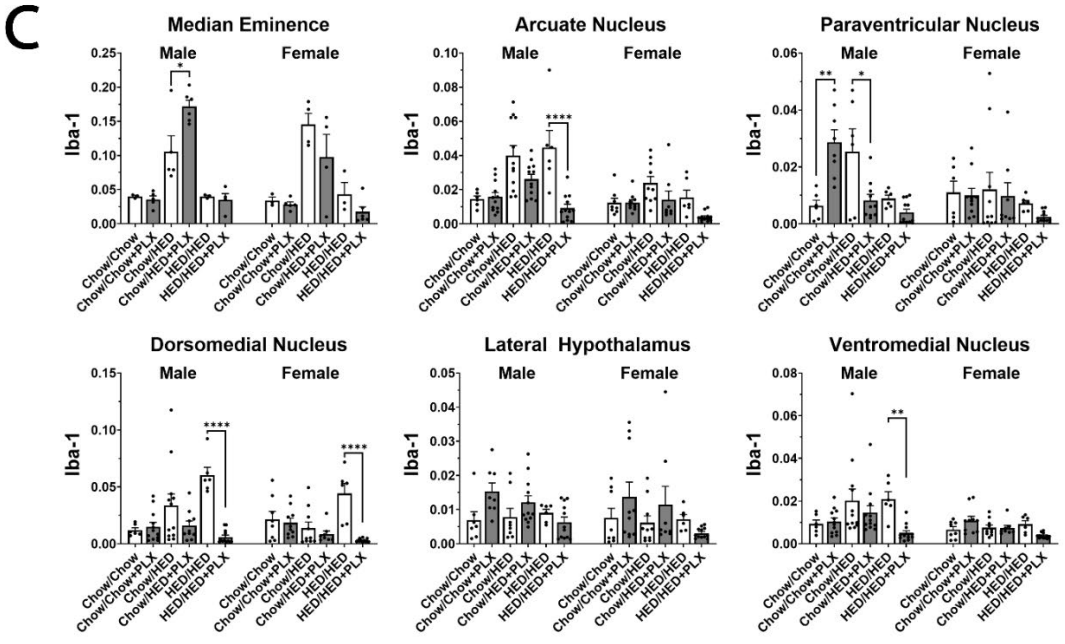

C
Median Eminence
Arcuate Nucleus
Paraventricular Nucleus
Dorsomedial Nucleus
Lateral Hypothalamus
Ventromedial Nucleus
Male
Female
Iba-1
Chow/Chow
Chow/Chow+PLX
Chow/HED
Chow/HED+PLX
HED/HED
HED/HED+PLX

Figure 2.5: PLX suppresses microglia activation in the hypothalamus.

(A-B) Representative coronal 20x images of Iba1 immunoreactivity in the hypothalamus near Bregma -1.92 mm with (A) Males and (B) Females. Inserts are 120x images from the same section demonstrating microglial morphology with each of the diet and treatment combinations. Male and female CD/CD, CD/CD + PLX, CD/HED, CD/HED + PLX inserts are from the paraventricular nucleus. Male and female HED/HED and HED/HED + PLX inserts are from the dorsomedial nucleus. Regions of interest (ROIs) are red = median eminence, blue = arcuate nucleus, yellow = ventromedial nucleus, orange = dorsomedial nucleus, white = periventricular nucleus, light blue = paraventricular nucleus, pink = lateral hypothalamic area. (C) Graphical representation of Iba1 intensity after 10 weeks of pre-anti-inflammatory treatment followed by 6 weeks of anti-inflammatory treatment. Scale bar = 500 μm in 20x images, 50 μm in 1200x images; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis was performed with Graphpad Prism 8.0 (GraphPad Software, Inc). One-way ANOVA was utilized for the statistical analysis of the binary area fraction of iba-1. The mean of each column was compared with the other columns using the Tukey's multiple comparisons test. Differences were considered significant at $p < 0.05$. Significance levels are reported as: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001. All samples were processed by an experimenter blind to the experimental condition.

2.4 RESULTS

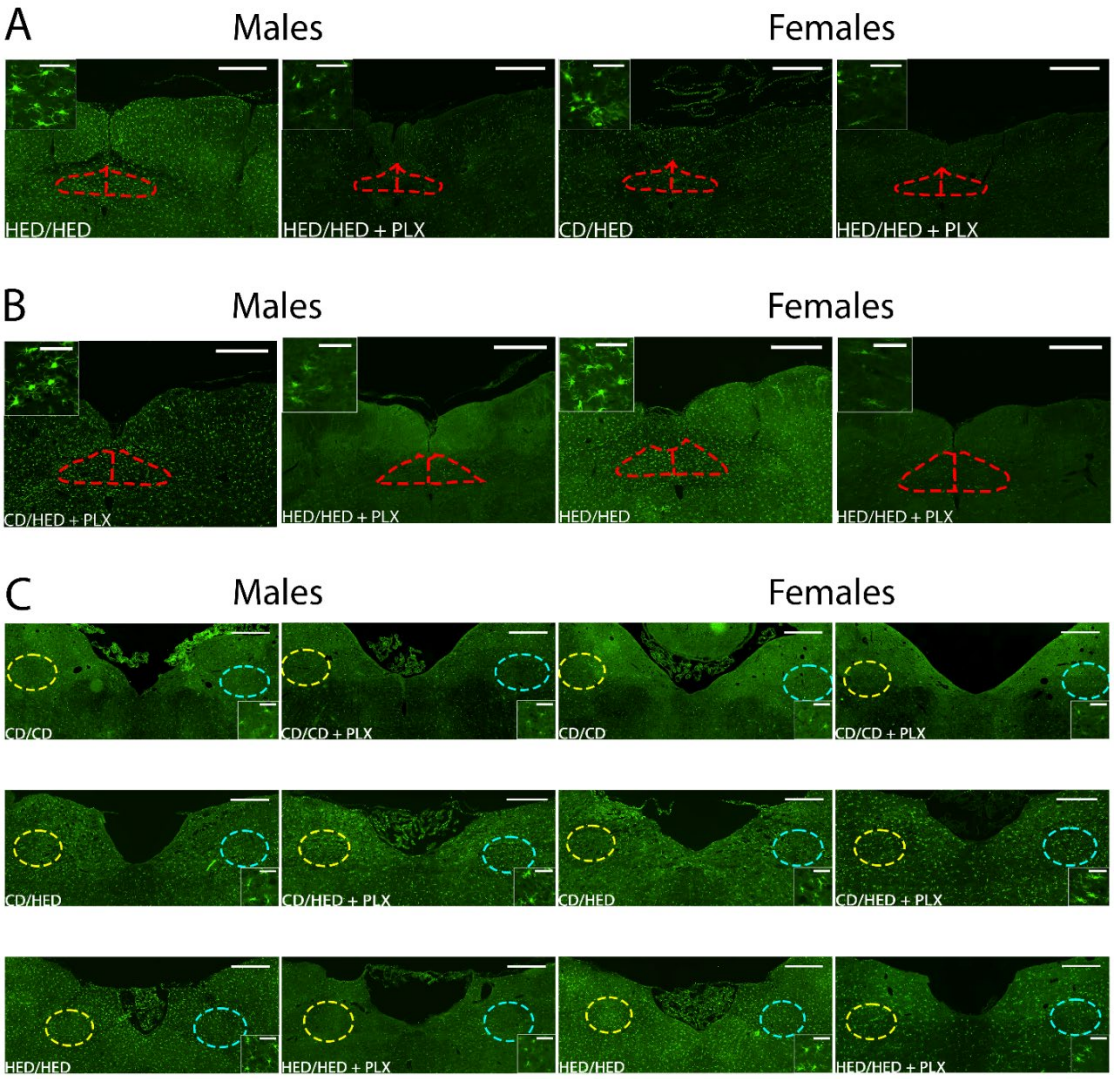
PLX Suppression of microglia activation in the hypothalamus

To examine PLX suppression of microglia activation in the CNS, we quantified the fluorescence of microglia in the hypothalamus and NTS in male (N=30) and female (N=27) Sprague Dawley rats fed CD, HFD, CD + PLX, or HFD + PLX. As predicted, PLX significantly suppressed microglia activation in the hypothalamus and NTS in rats that had been fed HFD. PLX significantly suppressed microglia activation in the arcuate nucleus, dorsomedial nucleus, and paraventricular nucleus in rats that had been fed HFD **(**Figure 2.5**)**. As expected, significantly increased levels of microglia activation were observed between HFD and CD groups. No significant effect of PLX was found in the periventricular nucleus.

PLX Suppression of Microglia Activation in the NTS

As predicted, PLX significantly suppressed microglia activation in all three regions of the NTS (rostral, intermediate, and caudal) in rats that had been fed HFD (figure 2.6). As opposed to the hypothalamus, there was consistent microglia activation between the left and right sides, but there were different degrees of significance across the three regions. PLX most significantly suppressed microglia activation in the rostral NTS. PLX significantly suppressed microglia activation after being fed HFD in the intermediate NTS in female rats while PLX significantly suppressed microglia activation after being fed HFD in the caudal NTS in male rats. Therefore, significance was reported for each region of the NTS, but not in both sexes for every region. No significantly suppressed microglia activation after PLX treatment and HFD was observed in the intermediate NTS in males or the caudal NTS in females. Though insignificant, these two regions did have

increased microglia activation in response to HFD compared to HFD treated with PLX. As expected, significantly increased levels of microglia activation were observed between HFD, and CD groups and no significance was found between CD and CD + PLX.



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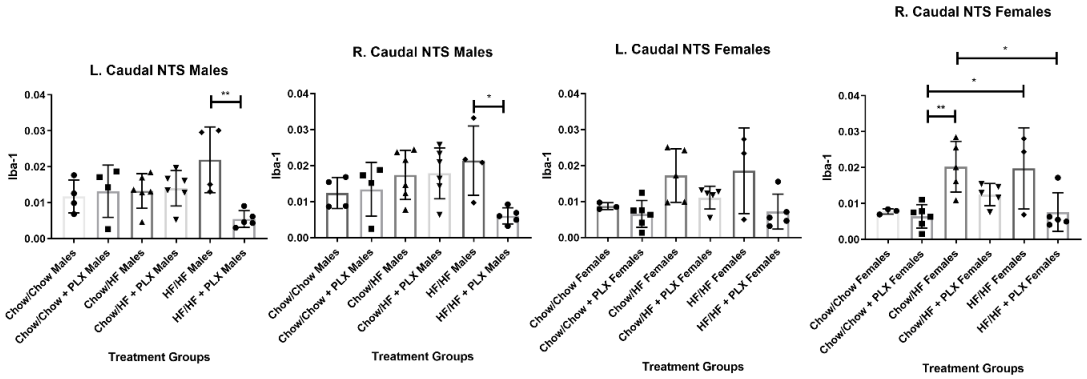
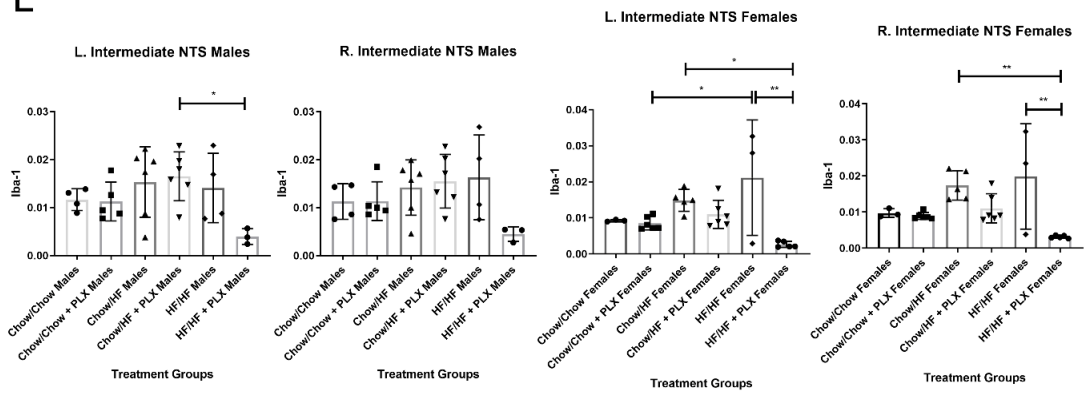

L. Caudal NTS Males
R. Caudal NTS Males
L. Caudal NTS Females
R. Caudal NTS Females
Iba-1
Treatment Groups
Chow/Chow Males
Chow/Chow + PLX Males
Chow/HF Males
Chow/HF + PLX Males
HF/HF Males
HF/HF + PLX Males
Chow/Chow Females
Chow/Chow + PLX Females
Chow/HF Females
Chow/HF + PLX Females
HF/HF Females
HF/HF + PLX Females

E


L. Intermediate NTS Males
R. Intermediate NTS Males
L. Intermediate NTS Females
R. Intermediate NTS Females
Iba-1
Treatment Groups
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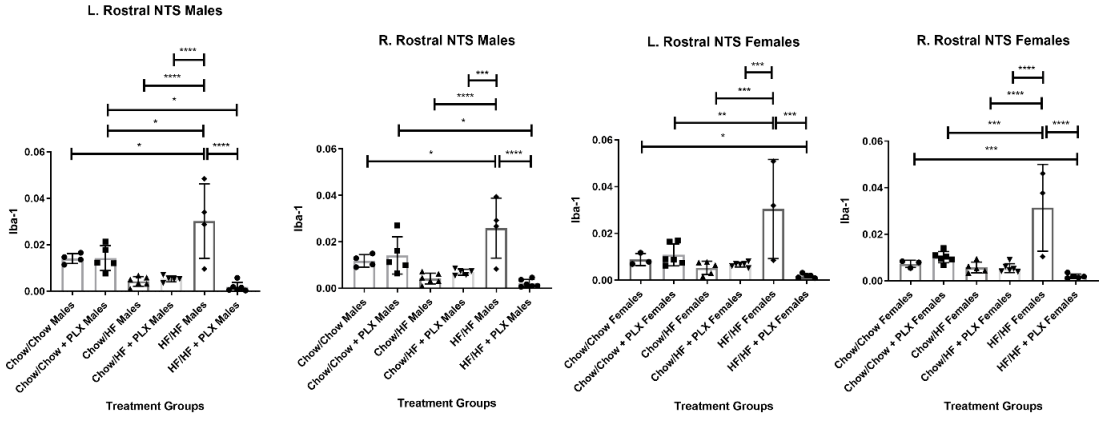
Chow/Chow + PLX Males
Chow/HF Males
Chow/HF + PLX Males
HF/HF Males
HF/HF + PLX Males
Chow/Chow Females
Chow/Chow + PLX Females
Chow/HF Females
Chow/HF + PLX Females
HF/HF Females
HF/HF + PLX Females

F


L. Rostral NTS Males
R. Rostral NTS Males
L. Rostral NTS Females
R. Rostral NTS Females
Iba-1
Treatment Groups
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Chow/Chow + PLX Males
Chow/HF Males
Chow/HF + PLX Males
HF/HF Males
HF/HF + PLX Males
Chow/Chow Females
Chow/Chow + PLX Females
Chow/HF Females
Chow/HF + PLX Females
HF/HF Females
HF/HF + PLX Females

Figure 2.6: PLX suppresses microglia activation in the NTS.

(A-F) Representative 20x images of Iba1 immunoreactivity in the caudal (A), intermediate (B), and rostral (C) NTS near Bregma -14.52 mm, -14.04 mm, and -12.6 mm respectively. Inserts are 120x (A-B) and 80x (C) images from the same section demonstrating microglial morphology with each of the diet and treatment combinations. Regions of interest (ROIs) are divided into left and right and are red (A-B) or yellow and blue (C). (D-F) Graphical representation of Iba1 intensity after 10 weeks of pre-anti-inflammatory treatment followed by 6 weeks of anti-inflammatory treatment. Scale bar = 500 μm in 20x images, 50 μm in 80x and 120x images; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

2.5 DISCUSSION

The increased levels of microglial cell activation in the ME, PeVN, AN, VMN, DMN, LHA, and PVN is indicative of inflammation in the brain associated with HFD as has been previously reported. The lower levels of microglial cell activation in these nuclei after treated with CD/HFD + PLX may be demonstrating a preventative or suppressing effect of PLX3397 as the inflammation in the brain was not triggered and the rats exhibited similar microglial activation levels compared to CD/CD and CD/CD + PLX treated animals in both males and females. The lower levels of microglial activation in these nuclei after given HFD/HFD + PLX may be demonstrating an additional reducing effect of PLX3397 as the inflammation in the brain was decreased compared to the higher levels of microglial activation observed in the rats given HFD/HFD. Lateralization of statistical significance was also observed in the right AN, right DMN, and right PVN. This

lateralization of hypothalamic inflammation and anti-inflammatory effect by PLX3397 may warrant further investigation in the impact of regional vagal innervation and communication to the brain in response to HFD and PLX3397. The increase of microglial cell activation exhibited in the PVN and LHA in the male animals is concerning and may also warrant further investigation into the effect of PLX3397 on non-obese animals eating a standard CD. In conclusion, PLX3397 may be an effective treatment option for individuals attempting to combat obesity in combination with other traditional strategies like exercise. Since we did see positive results of a decrease in levels of microglia activation in rats fed HFD and treated with PLX3397, our study gives hope that the drug may be administered to help patients with cognitive/neurological disorders as previously mentioned, but more studies will need to be conducted.

Acknowledgements

This work is our laboratory's contribution to a larger project in collaboration with and will be submitted by Dr. Patricia M. Di Lorenzo in Binghamton University, New York. Dr. Di Lorenzo and the members of her lab conducted the animal experiment and collected all the tissues. The brains were collected and sent to our lab for sectioning, immunohistochemistry, imaging, and quantification. Dr. Czaja, Dr. Minaya, and their undergraduate students Noah Weinstein, Johnathan Vainer, Brandon Garten, Sakshika Vakiti, Olivia Johnson, Mandy Dao, Gabriel Grosswald, and Rushabh Sheth were instrumental in teaching me and helping me process the tissues. Thank you.

CHAPTER 3

IMPACTS OF PRENATAL SURGICAL STRESS AND MATERNAL HIGH FAT DIET ON METABOLIC DYSFUNCTION IN DIET-INDUCED OBESE AND DIETARY RESISTANT OFFSPRING AFTER SHORT TERM HIGH FAT FEEDING

Brent Norwood, Priya Balasubramanian, Katrina Linning, Andrew Shin, Pratibha A. Varde, Puliyur S. Mohankumar, Sheba Mohankumar. To be submitted to *International Journal of Obesity.*

3.1 ABSTRACT

The role of stress and High-fat diet (HFD) consumption on the development of obesity has been widely investigated. However, the combined effect of acute prenatal stress and maternal HFD consumption has not been investigated and the mechanisms by which these two factors promote obesity in the offspring is not fully understood. To measure the effects of surgical prenatal stress, diet-induced obese (DIO) and dietary resistant (DR) dams were subjected to jugular catheterization once a week for three weeks and a 1ml blood sample was collected. They were fed a chow or HFD for the duration of pregnancy. The offspring were weaned onto a chow diet (CD) at 3 weeks of age and caloric intake and body weight was measured every week. At 9 weeks of age, the offspring were placed on HFD for 1 week at the end of which they were euthanized by decapitation. Trunk blood was collected, and serum was separated. Fat was harvested from the carcass and weighed. Corticosterone and leptin levels were measured in the serum by radioimmunoassay. Cytokine levels were measured in maternal serum using a multiplex assay. The results indicate that altered normal fetal programming due to maternal surgical stress in combination with maternal HFD contributed to changes in food intake, body weight, corticosterone levels, fat levels, and leptin that may work in conjunction to promote obesity.

3.2 INTRODUCTION

Obesity continues to be a growing health problem around the world. Maternal obesity poses a threat, not only to the mother, but to the health of the offspring as well. Obese women are at risk of pregnancy complications such as preeclampsia, gestational diabetes mellitus, and cesarean delivery [120, 121]. Moreover, there is a greater chance for obese moms to have children with obesity. For every two pounds of excess weight, an obese mom increases the chance of having an obese child by 4.5% [122]. Unfortunately, childhood obesity has been growing at an alarming rate of 15% over the past few years (CDC). Therefore, it is important to understand the prenatal factors that contribute to the development of obesity in the offspring.

Prenatal stress is a well-established factor that is known to play a role in the development of obesity in offspring [123]. Several maternal factors can contribute to the increased stress during pregnancy. These include maternal undernutrition or overnutrition, smoking, maternal diabetes and hyperglycemia, and obstetrical complications such as pre-eclampsia and exposure to endocrine disruptors [124]. Like maternal HFD consumption, prenatal stress can affect fetal development by intra-uterine growth retardation (IUGR) leading to offspring with low birth weight. Later in life, the same offspring have the propensity to develop obesity rapidly after HFD exposure [125]. Restraint stress has been frequently used in rodents as a paradigm for prenatal stress for over 30 years to study its effects on neurobehavioral outcomes in offspring [126]. Noise is used as another stressful stimulus during pregnancy [127]. In this study we used jugular catheterization, a minimally invasive surgical procedure that is regularly used for blood sampling as the stressful stimuli. This was done to obtain blood samples from pregnant dams once a week

during the 3 weeks of pregnancy in an attempt to identify potential "programming" factors that may play a role in the development of obesity in DIO but not DR rats.

The diet-induced obese and dietary resistant (DIO/DR) rat model was used in these studies since they are like humans in how they are prone to the development of obesity. They were developed by Levine et al by repeated selective breeding of rats that gained weight or not when placed on a high fat diet. The two strains have a Sprague Dawley background but differ only in their capacity to gain weight or not after exposure to HFD [128]. Although this trait is believed to be polygenic, we wanted to use this model to determine the underlying cause(s) of obesity especially in the context of prenatal stress.

High-fat diet (HFD) has been established to be a cause of obesity, but the mechanisms in the multiple body systems it affects have yet to be fully understood. HFD can cause obesity in adults, but it can also detrimentally affect fetal development leading to decreased birth weight and induce metabolic disorders from childhood and potentially into adulthood [121]. Exposure to HFD has been shown to activate the hypothalamo-pituitary adrenal axis or "stress axis" which is made up of corticotrophin-releasing hormone (CRH) neurons in the hypothalamus, corticotrophs in the anterior pituitary gland, and the adrenal which secretes corticosterone (CORT). Norepinephrine (NE), the activates the stress axis causing downstream effects on CRH, adrenocorticotrophic hormone (ACTH) and CORT levels. The hypothalamus plays a major role in regulating multiple bodily functions including feeding regulation. HFD has also been shown to cause a low-grade inflammatory state within the CNS, potentially disrupting neurotransmitter signaling

within the brain [129]. HFD also promotes hyperphagia quickly and can cause significant body weight increase within 3 weeks [130].

The combined effects of prenatal surgical stress and maternal HFD consumption have not been studied before. We hypothesize that prenatal stress would alter feeding circuits in the brain and increase the propensity of DIO rats to gain more weight than DR rats. Exposure to HFD would further aggravate this condition. The offspring were challenged with HFD in adulthood to determine the potential effects of prenatal programming with stress and HFD.

3.3 MATERIALS AND METHODS

Animal treatment

Animal Care. Adult male and female DIO and DR rats were purchased from Charles River laboratories (Wilmington, MA). Upon arrival, the animals were given time to acclimate to their new environment and housed in air-conditioned rooms (23 ± 2°C) with a 12-hour light/dark cycle with *ad libitum* access to feed and water. Females were placed on a chow (CD) or HFD 1 week before breeding and throughout gestation. CD (23% protein, 72% carbohydrate, and 5% fat) had a caloric content density of 3.11 kcal/g and HFD (20% protein, 35% carbohydrate, 45% fat) a caloric content density of 4.73 kcal/g. Males and females were paired and housed together overnight when the females were determined to be in proestrus. The next morning, vaginal smears were performed to check for sperm and to confirm copulation had occurred. If sperm was found, that day was considered day 1 of gestation and incremental body weight increase was also used to confirm pregnancy by weekly body weight checks. If pregnancy could not be confirmed, the rats were excluded from the study. DIO (n=8) and DR (n=8) dams were subdivided

into the following groups: DIO control (normal pregnancy), DIO prenatal stress (surgical stress during pregnancy), DR control, and DR prenatal stress. The dams in the prenatal stress groups were subjected to jugular catheterization once a week for the duration of the pregnancy (3 times during the entire pregnancy) and blood samples (1 mL) were collected to assess changes in adiponectin, leptin, IL-1beta, IL-6 and TNF alpha.

Dams were anesthetized with isoflurane, shaved on the ventral surface of the neck, disinfected, and a 2 cm long incision was made on the right side of the neck. The jugular vein was isolated by blunt dissection, 2 retention sutures were placed under the jugular vein and a small cut was made in the vein using a sterile 18-gauge needle. A sterile silastic catheter was then inserted into the jugular vein and 1 mL of blood was collected, which was replaced with saline. The catheter was removed slowly while pressure was applied on the vein to prevent any further bleeding. After making sure the bleeding had stopped, the incision was stapled. The control dams were left undisturbed for the duration of their pregnancy.

Treatment of offspring

Offspring birth weight was recorded within 24 hours of birth. Litter sizes were normalized for consistency between groups and to ensure consistent nutrition from the mothers of different groups. Eight pups per mother were selected to be included in the study (4 male and 4 female). The offspring were weaned onto a chow diet (CD) at 3 weeks of age. Caloric intake was monitored weekly for the next 6 weeks. Caloric intake (means ± SE, kcal) was calculated by measuring daily food intake for each week and multiplying it by the CD caloric content (3.11 kcal/g). Animals were placed in individual cages on the day of food intake measurement. A known quantity of food was added to the

bin. The amount of food left over was weighed and the food intake recorded on the following day. Body weight was measured at weekly intervals from 3 weeks to 8 weeks of age.

At 9 weeks of age, the offspring from each mother were housed individually and fed HFD for 1 week before euthanasia by decapitation. These methods were done in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at Michigan State University. A schematic depicting the procedure is provided in fig 3.1.

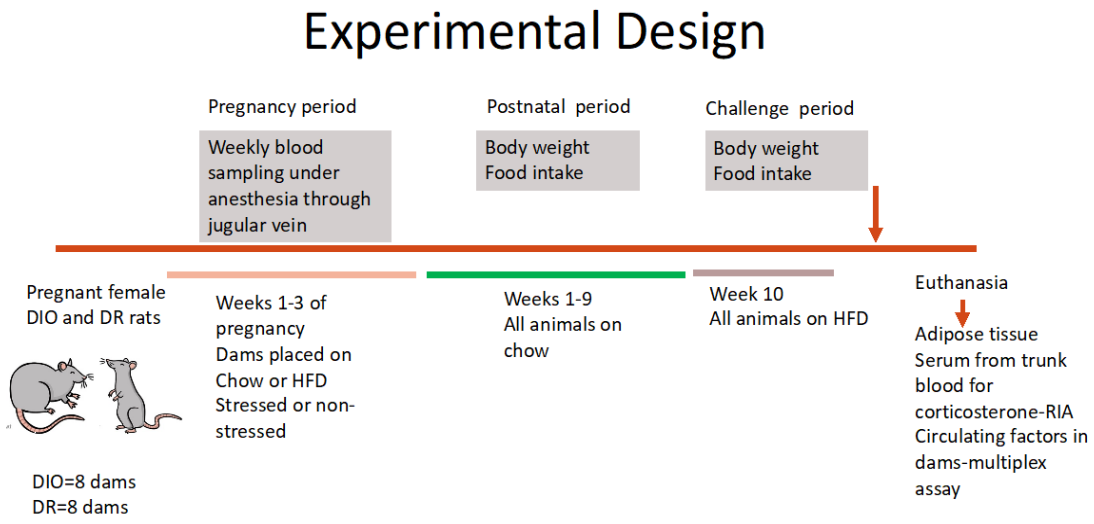

Figure 3.1: Experimental Design.

Pregnant DIO and DR rats (N=8) were evenly divided (N=4) into prenatal stress and non-stress (control) groups. The prenatal stress group underwent jugular catheterization once a week for 3 weeks. They were fed either a chow or HFD for the duration of gestation. After birth, offspring from each prenatal programming group were weaned off their

mothers at 3 weeks of age and fed chow diet (CD) for 6 weeks. After 9 weeks of age, the offspring were challenged with a high-fat diet (HFD) for 1 week. After 1 week of postnatal treatment, the offspring were euthanized by decapitation and the brains were collected and stored at -80°C until processing. Abdominal fat that included fat attached to the viscera, abdominal wall and the kidneys was harvested from the carcass and weighed. Trunk blood was collected, serum separated and analyzed for hormone measurements using RIA.

Corticosterone and Leptin RIA and multiplex for cytokines

Serum from trunk blood was subjected to a double antibody RIA as described previously [131]. Samples were assayed in duplicate. The intraassay coefficient of variation was less than 5%. Serum from weekly blood samples collected from the dams were subjected to a multiplex assay using a Magpix Luminex machine. Multiplex kit (Millipore Sigma) was used to determine the levels of adiponectin, IL-1 beta, IL-6 and TNF-alpha simultaneously using 50ul of the serum sample in duplicate.

Statistical Analysis

GraphPad Prism Software (9.4.0), Inc. was used for statistical purposes. Data were expressed as mean ± SEM. Weekly food intake was analyzed using repeated measures ANOVA followed by Fisher's LSD post hoc test. Average caloric intake during the last week were analyzed using three-way ANOVA followed by Šídák's multiple comparisons test to compare interactions between genotype, stress, and diet. Similarly, differences in Leptin, corticosterone, and fat weight were analyzed using 3-Way ANOVA followed by Fisher's LSD test. Differences in cytokine levels in maternal serum was

determined using 3-way repeated measures ANOVA followed by a posthoc test. Šídák's multiple comparisons test was used to determine the effect of genotype, stress and diet and their interaction. A p-value <0.05 was considered statistically significant but only p<0.01 is reported here.

3.4 RESULTS

Changes in circulating factors in pregnant dams during the 3 weeks of pregnancy

There were marked changes in the levels of IL-6, IL-1beta, adiponectin, and TNF alpha between DIO and DR moms during the 3 weeks of pregnancy. Within each group, there were changes in the levels of these cytokines/adipokines from week to week. While most of the cytokines were moderately elevated in chow fed animals, HF feeding markedly reduced the levels of many of these cytokines. The levels in DIO animals were very different from that in DR rats.

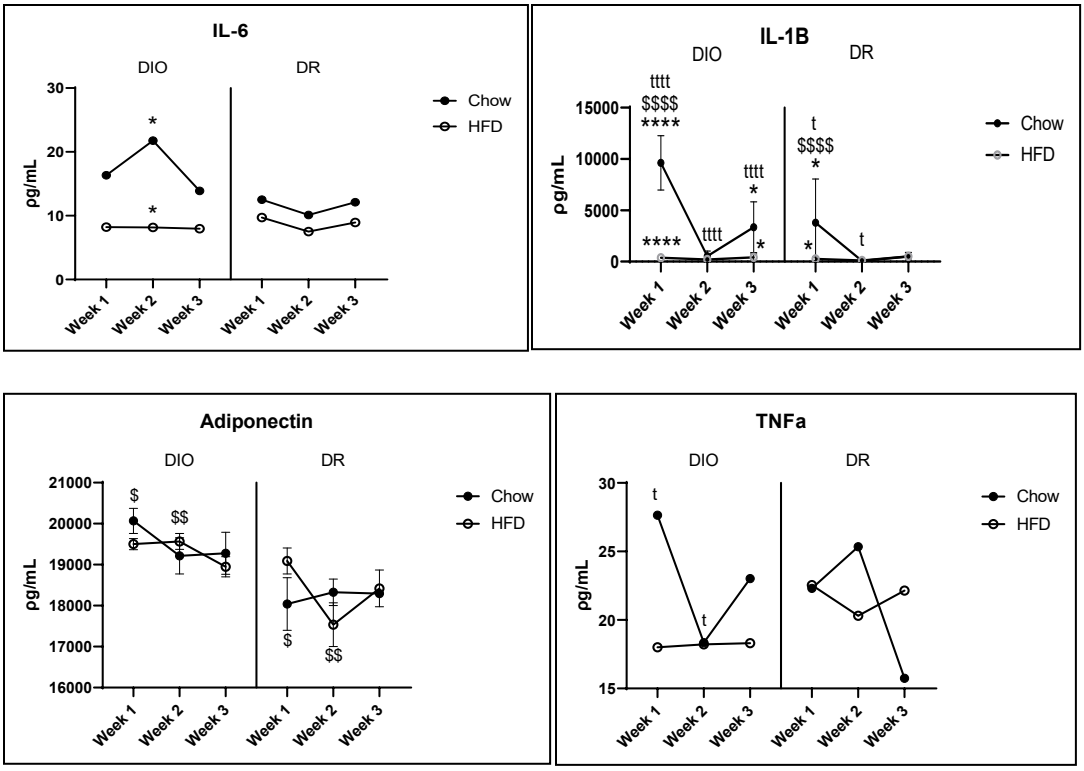

Figure 3.2: Changes in circulating levels of cytokines and adipokines in the dams during the 3 weeks of pregnancy.

There were significant changes in the levels of adiponectin (top left), TNF alpha (top right), IL-1 beta (bottom left) and IL-6 (bottom right). Significant differences ($p<0.05$) are indicated. $ indicates genotype differences, * indicates a diet difference and t indicates differences with time.

Effects of prenatal stress on offspring body weight post weaning

DIO offspring appeared to gain the most body weight after weaning. Interestingly, DIO chow fed animals that were subjected to prenatal stress gained the most weight followed by non-stressed chow fed DIO offspring. DIO moms born of stressed, HFD fed

dams had markedly lower body weights. This pattern was maintained in both males and females.

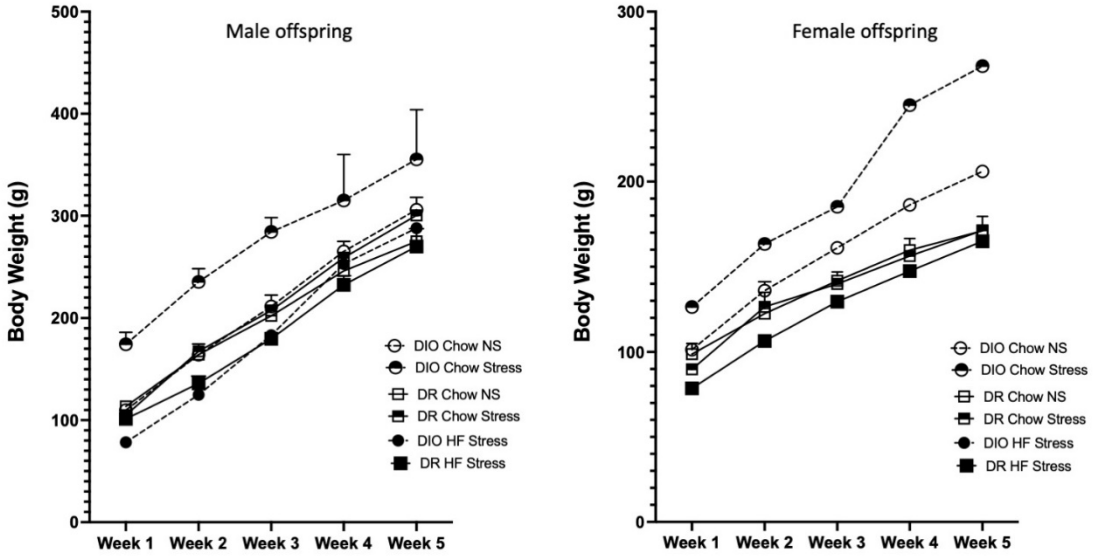

Figure 3.3: Body weight changes in offspring born from stressed and non-stressed DIO and DR dams that were fed a chow or HFD.

DIO chow fed offspring gained weight more than any other group in both males and females.

Effects of Prenatal Stress and maternal HFD on food intake in male and female DIO and DR offspring

The effect of prenatal stress and maternal HFD on food intake in the male and female offspring was analyzed by measuring the average food intake after the animals had been fed HFD for 1 week. A significant genotype effect as well as a combined diet and genotype effect ($P<0.0001$; $P=0.0053$) decreasing food intake between females was

observed. This effect is depicted in figure 3.4 where significant differences between the DIO/DR non-stressed/stressed animals born of moms fed HFD (96.41 ± 17.39; 82.39 ± 18.1) are shown. A significant genotype and a separate stress effect (P<0.0001; P=0.0093) decreasing food intake in male rats was observed although Šídák's multiple comparisons did not report strong significance between groups.

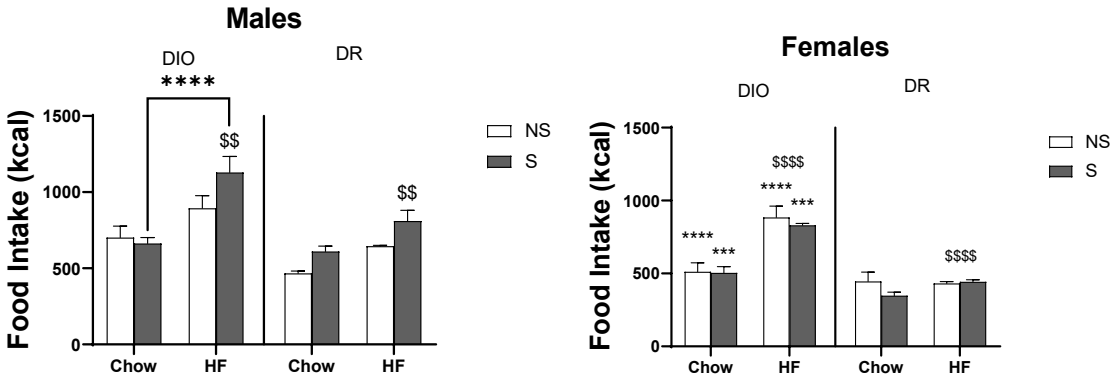

Figure 3.4: Food intake in DIO/DR offspring subjected to surgical prenatal stress and postnatal CD or HFD challenge in adulthood.

Food intake was measured every day for each animal during the week of HFD exposure. The weekly average caloric intake was analyzed using three-way ANOVA followed by Fisher's LSD test. Multiple comparisons between groups controlling for one factor at a time was done using Šídák's multiple comparisons test. Prenatal surgical stress had no significant effect on caloric intake. However female DR groups exposed to HFD during pregnancy consumed less calories compared to the DIO groups. Graphs represent mean ± SEM from 6-9 offspring per treatment group. $$$ represents a significant difference between genotype: DIO vs DR ($p \leq 0.001$); $$$$ represents p<0.0001.

Effects of Prenatal Stress and Maternal HFD on body weight in male and female DIO and DR offspring

Body weight of offspring was measured at the end of 1 week of exposure to HFD. Multiple treatment effects were observed including a diet, genotype (DIO vs DR), and stress effect in the male animals as well as a diet, genotype, and combined diet and genotype effect in the females. These comparisons can be visualized in figure 3.5 where DIO rats were significantly heavier than DR rats in every treatment group in both males and females. As expected, the males weighed more than the females, but there was no body weight difference between the stressed and non-stressed groups. A diet effect was apparent between DIO stressed females born of CD and HFD fed dams. Overall, every DIO group was significantly heavier than the corresponding DR group in both sexes (Fig 3.3).

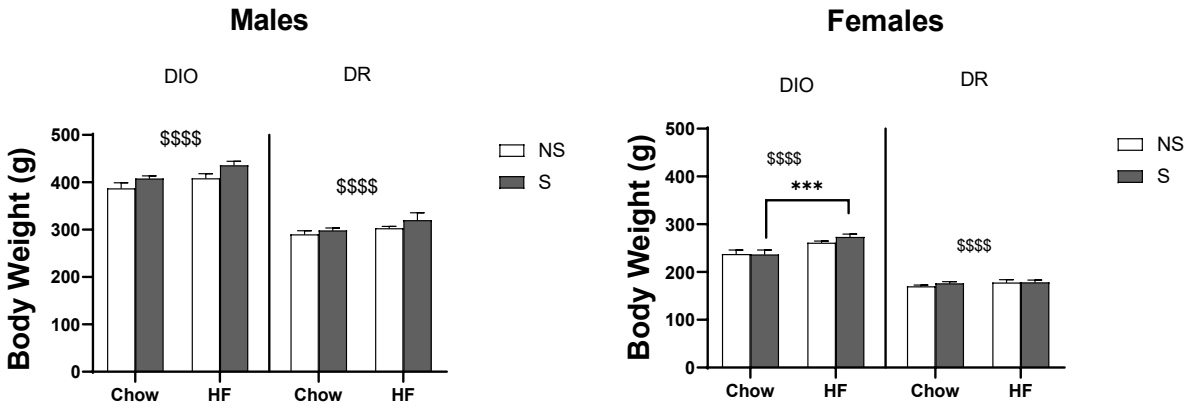

Figure 3.5: Body weight in DIO/DR offspring subjected to prenatal surgical stress and postnatal CD or HFD.

Body weight was measured at the end of 1 week of HFD exposure. The weekly average body weight was analyzed using three-way ANOVA followed by Fisher's LSD test.

Multiple comparisons between groups controlling for one factor at a time was done using Šídák's multiple comparisons test. Prenatal surgical stress increased body weight only in DIO females when they were challenged with HFD. Overall DIO rats weighed more than DR animals. Graphs represent mean ± SEM from 6-9 offspring per treatment group. $$$$ represents a significant difference between genotype ($p<0.0001$). *** represents a significant diet effect ($p<0.001$).

Effects of Prenatal Stress and maternal HFD on corticosterone levels in male and female DIO and DR offspring

Corticosterone levels in the different treatment groups are shown in figure 3.6. In the males, there was a significant diet, genotype, stress, and combined diet and genotype effect. In the females, there was a combined diet and stress as well as a combined diet, genotype, and stress effect. Corticosterone levels increased significantly with stress in DIO males whose moms were exposed to HFD. HFD diet treatment of moms increased corticosterone levels significantly in DR males. In contrast, DIO females had a diet effect with respect to corticosterone. Moreover, DR females born of moms fed HFD had higher levels of corticosterone. Stressed DIO males had higher levels of corticosterone compared to DR males. However only non-stressed females born of HFD fed moms demonstrated a similar genotype difference.

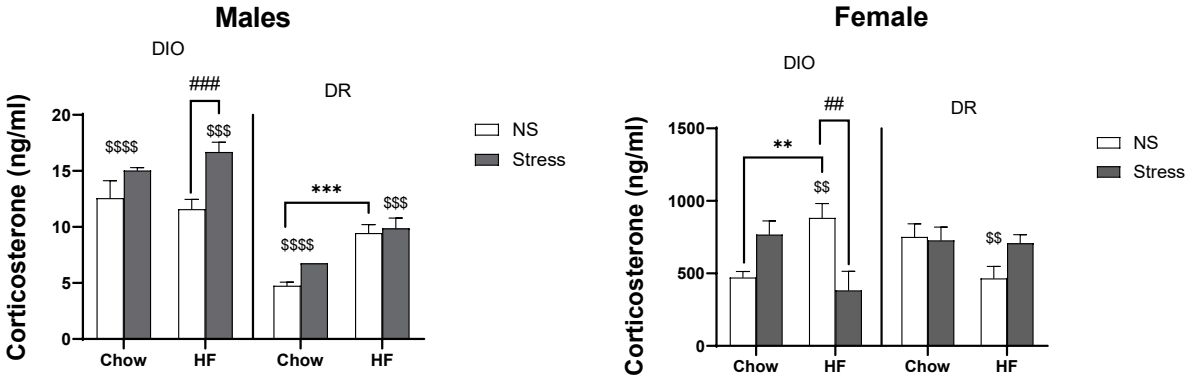

Figure 3.6: Corticosterone in DIO/DR male and female rats after prenatal stress and postnatal CD or HFD consumption.

Serum corticosterone was measured in trunk blood collected after sacrifice. The corticosterone levels were analyzed using three-way ANOVA followed by Fisher's LSD test. Multiple comparisons between groups controlling for one factor at a time was done using Šídák's multiple comparisons test. Prenatal surgical stress increased corticosterone only in DIO males that were born from HFD fed dams. In contrast, prenatal stress reduced corticosterone levels in DIO females born of HFD fed dams. A diet effect was apparent in DR males and DIO females. A genotype effect was more pronounced in males than females. Overall, corticosterone levels were several folds higher in females than males. Graphs represent mean ± SEM from 6-9 offspring per treatment group. "*" represents diet differences, "$" genotype, and "#" prenatal stress effects. ** indicates $p<0.01$, *** $p<0.001$ and ****$p<0.0001$.

Effects of Prenatal Stress and maternal HFD on abdominal fat accumulation in male and female DIO and DR offspring that were challenged with 1 week of HFD

Abdominal fat was harvested from the carcass and weighed. Prenatal stress and HFD produced significant effects on the fat levels as shown in figure 3.7. There was a significant diet, genotype, and a modest stress effect in males as well as a genotype and combined diet, genotype, and stress effect in females. DR animals had less abdominal fat accumulation than DIO animals. Prenatal surgical stress only increased fat deposition in DIO males born from moms fed HFD. DR non-stressed males had a diet effect increasing fat deposition when their moms were fed HFD.

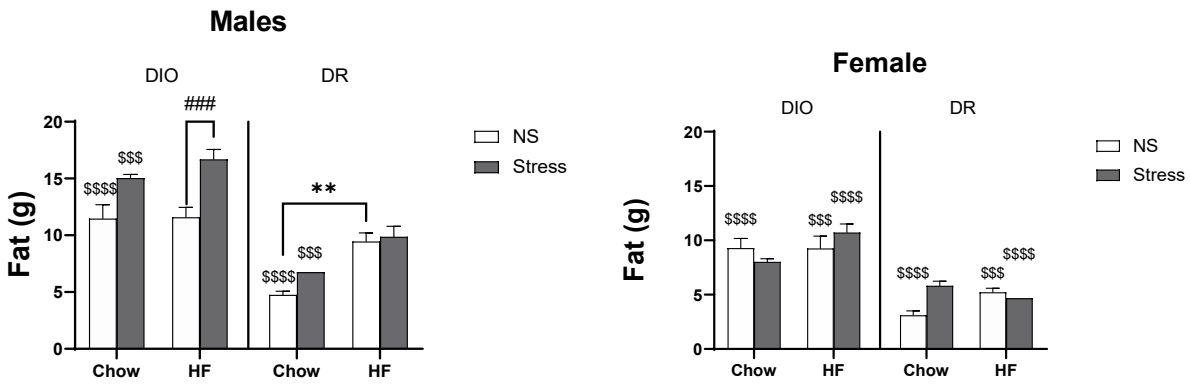

Figure 3.7: Abdominal fat accumulation in DIO/DR offspring after prenatal surgical stress and maternal HFD consumption.

Abdominal fat was harvested after sacrifice and weighed. The fat weights were analyzed using three-way ANOVA followed by Fisher's LSD test. Multiple comparisons between groups controlling for one factor at a time was done using Šídák's multiple comparisons test. Prenatal surgical stress increased abdominal fat deposition only in DIO males when

they were challenged with HFD. A diet effect was apparent in non-stressed DR males. A genotype effect was more pronounced in males than females. Overall, abdominal fat deposition was higher in DIO than DR offspring. Graphs represent mean ± SEM from 6-9 offspring per treatment group. "*" represents diet differences, "$" genotype, and "#" prenatal stress effects. ** indicates p<0.01, *** p<0.001 and ****p<0.0001.

Effects of Prenatal Stress and maternal HFD on leptin levels in male and female DIO and DR offspring challenged with HFD for 1 week

Leptin levels were measured in the trunk blood using RIA and are depicted in figure 3.8. A significant genotype effect was apparent only in non-stressed, males born of chow fed moms. DR females had lower leptin levels overall compared to DIO females. Prenatal stress further reduced leptin levels in DR females that were subjected to prenatal stress and whose moms were placed on HFD. There was no effect of maternal diet in males or female offspring.

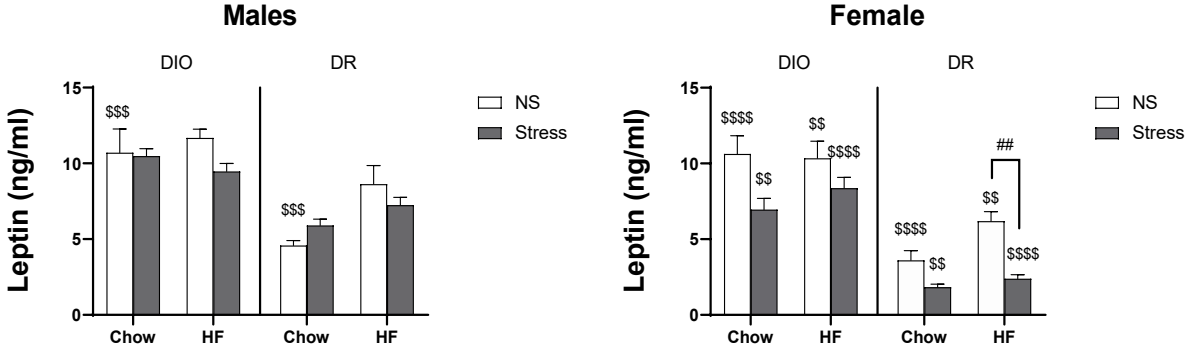

Figure 3.8: Leptin in DIO/DR male and female rats after prenatal stress and maternal HFD consumption.

Serum leptin was measured in trunk blood collected after sacrifice. Leptin levels were analyzed using three-way ANOVA followed by Fisher's LSD test. Multiple comparisons between groups controlling for one factor at a time was done using Šídák's multiple comparisons test. Prenatal surgical stress reduced leptin levels only in DR females when they were challenged with HFD. DIO rats had more leptin levels compared to DR rats. No maternal diet effect was noted. Graphs represent mean ± SEM from 6-9 offspring per treatment group. "$" represents genotype and "#" prenatal stress effects. ** indicates $p<0.01$, *** $p<0.001$ and ****$p<0.0001$.

3.5 DISCUSSION

Earlier studies have clearly established that there is a prenatal programming effect on postnatal development for adult diseases such as obesity [132-135]. Therefore, obesity is a condition that can be "programmed" in the developing fetus using adverse stimuli such as reducing protein intake in dams [136, 137], subjecting them to stressful paradigms [123, 138], or feeding them high energy diets [139, 140]. In addition to prenatal stress programming, studies have also been conducted to show that HFD induces obesity at a high rate or in a short period of time [141, 142]. These studies have typically been conducted using males in animal models to avoid the confounding effects of the estrus cycle. In this study, we have shown that prenatal surgical stress in combination with HFD may cause changes in the levels of corticosterone, leptin, and fat to increase food intake and body weight when challenged with HFD in adulthood. DIO males had higher levels

of corticosterone compared to DR males and their abdominal adipose tissue was also significantly higher falling in line with the ability of corticosterone to support fat deposition in the abdomen [143, 144]. Interestingly, in prenatally stressed DIO males, corticosterone levels increased when moms were fed HFD, but in females, prenatal stress increased corticosterone levels in chow fed animals but decreased when the moms were fed HFD. Therefore, in males, stress levels remained high in DIO animals even when the moms consumed HFD but in females stress levels decreased after moms consumed HFD. The reason for this diametrically opposite effect could be attributed to the effect of leptin. Leptin levels increase with adiposity, but leptin can also suppress the stress axis leading to a reduction in corticosterone levels [145, 146]. This could be the reason why HFD suppresses corticosterone levels in females. The reason for the persistent elevation in corticosterone levels in males despite the increase in leptin levels could be attributed to leptin insensitivity [147] or sequestration of leptin by triglycerides that prevent its passage across the blood brain barrier to suppress the HPA axis [148]. Besides leptin, other hormones such as resistin can increase expression of SOCS-3 in the hypothalamus leading leptin resistance/insensitivity [149]. This phenomenon is suspected to involve epigenetic mechanisms [150]. The difference in leptin resistance between males and females needs to be further explored.

To investigate if there are any major changes to feeding behavior, we measured and analyzed the caloric intake data from the animals in our study and did not find any statistically significant differences. Although there appears to be a trend for DIO rats to consume more, the impact is more clearly apparent in the body weight of these animals and their abdominal fat deposition. In contrast to males, female DIO rats consumed more

than DR rats when their moms were placed on HFD. Prenatal stress had no impact on food consumption in both groups. Interestingly, DR females whose moms were placed on the HFD consumed less food compared to the corresponding DIO rats. This could suggest a possible genetic influence in DR females that prevents them from consuming more.

A high fat diet can play a role in the accumulation of body fat and can lead to the development of obesity. Stress has also been shown to promote obesity, therefore an accumulation of body fat. In this study, we also show that, especially in males, the combination of stress and HFD leads to increased fat accumulation in DIO and DR rats with a more significant effect in DIO animals. It appears that stress plays a significant role in the accumulation of abdominal fat in DIO rats regardless of their diet. However only DIO females whose moms were fed HFD had more fat deposition. This indicates that prenatal stress in combination with HFD may promote fat accumulation eventually leading to the development of obesity.

Leptin, the hormone that is important for its role in limiting food intake by inhibiting hunger may also be altered in this prenatal stress and maternal HFD interaction. In a healthy animal or individual, leptin is responsible for preventing hunger when extra food or energy is not required [151]. DIO animals both male and female had higher levels of leptin as expected compared to their DR counterparts. Interestingly, prenatal stress reduced leptin levels in DR females whose moms were placed on HFD. However, their abdominal fat deposition was comparable to the other DR groups. This would indicate the prenatal stress directly programs the adipose tissue of DR rats to produce less leptin and may be makes the hypothalamus refractory to hunger signals because their food intake is

unchanged. Leptin levels were even lower in all the DR animals, but that is logical as they were the animals that consumed the least amount of food.

As body fat levels increase, it might be assumed that body weight would increase as well. This does seem to be true for the offspring in our study but, within the same genotype, there weren't significant differences. It could be that, although fat accumulation has been shown to increase at least in DIO HF male and females, there could be other changes in the body composition as well that have not been investigated. It is also worth noting that body weight was lower in every DR group compared to the matched DIO group confirming the DIO/DR animal model used in our study.

In conclusion, our study reveals that prenatal stress programming (stress to the dams) along with feeding moms HFD results in specific changes in food intake, body weight, corticosterone levels, fat levels, and leptin that may work in conjunction to promote obesity in the offspring. It is likely that two insults (stress to the mother and HFD) impact different tissues and processes as they were delivered at different time points when tissues were at different stages of development. Further investigation is required to understand the underlying mechanisms that might be involved in this complex process of obesity development.

CHAPTER 4

IMPACT OF PRENATAL SURGICAL STRESS AND MATERNAL HIGH FAT FEEDING ON NEUROENDOCRINE RESPONSES TO HIGH FAT FEEDING IN DIET-INDUCED OBESE AND DIETARY RESISTANT RATS

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4.1 ABSTRACT

It is well known that stress or exposure to a high fat diet (HFD) can contribute to the development of obesity. However, the combined effects of prenatal surgical stress and maternal HFD consumption have not been investigated and the mechanisms by which these two factors promote obesity in offspring when they are challenged with HFD are not fully understood. To assess the impact of prenatal surgical stress, diet-induced obese (DIO) and dietary resistant (DR) dams were subjected to jugular catheterization for blood sampling once a week during the three weeks of gestation. Body weight of the dams was recorded every week. Body weight of the offspring was measured within 24 hours of birth. The offspring were weaned on chow diet (CD) at 3 weeks of age and food intake was measured. After 9 weeks of age, the offspring were fed HFD (45% calories from fat) for 1 week. The offspring were euthanized by decapitation, their brains frozen and sectioned. Specific hypothalamic and other brain nuclei were isolated and analyzed by HPLC for monoamine neurotransmitter concentrations. There were region and neurotransmitter-specific alterations in the hypothalamic nuclei that are involved in the regulation of feeding behavior that was diametrically opposite to the changes observed in hypothalamic nuclei regulating stress. Overall, norepinephrine and dopamine appeared to be affected more compared to serotonin. These changes were also sex- and genotype specific. These results suggest that changes in neurotransmitter levels in specific hypothalamic nuclei in response to prenatal stress could contribute significantly to altered feeding behavior. These effects are confounded by predisposition to obesity, sex, and diet.

4.2 INTRODUCTION

Pregnancy is a critical period for the development of the offspring. Changes in the health of the mother during pregnancy or exposure to adverse conditions can impact the offspring leading to long term effects. Obesity is one such condition that has roots in prenatal development, but the underlying mechanism are yet to be fully understood [11]. As shown in previous studies, one of the major contributors to the development of obesity is the consumption of a high-fat diet (HFD) [129, 152-154]. In addition to HFD, studies have shown prenatal stress plays a role in the development of obesity in offspring. Prenatal stress has been observed to cause intra-uterine growth retardation (IUGR) which leads to offspring with low birth weight. The same offspring with lower birth weight, when exposed to HFD in later life, gain weight quickly, leading to the development of obesity [125]. A diet-induced obese and dietary resistant (DIO/DR) rat model has been used in studies in which DIO offspring gain weight more quickly than the DR offspring, demonstrating the development of obesity quickly in the DIO juveniles [128]. Therefore, investigating the health and healthy habits of pregnant mothers, especially their diet, could be a strategy to combat the constantly rising rate of obesity in the US and around the world.

The exact mechanism by which HFD and stress promote obesity is not fully understood. HFD is considered a "stressor" in which chronic exposure to HFD has been shown to activate the "stress axis" or the hypothalamo-pituitary-adrenal (HPA) axis. The HPA axis is made up of corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus, the anterior pituitary gland, and the adrenal cortex which secretes corticosterone (CORT). Norepinephrine (NE) that is generated by specific populations of neurons in the brain stem, stimulates CRH neurons in the

PVN, thus activating the HPA axis. This activation of the HPA axis therefore can be quantified by measuring levels of norepinephrine (NE) in the paraventricular nucleus of the hypothalamus as well as tyrosine hydroxylase (TH), the rate limiting enzyme for the synthesis of NE and dopamine (DA). While significant changes in levels of NE, CRH, TH, and CORT have been observed between DIO and DR rats that were chronically exposed to HFD, it is unclear whether these changes are a direct cause of HFD or obesity itself [154].

The hypothalamus is a crucial region of the brain that is made up of many smaller nuclei, some of which play a critical role in metabolism and feeding regulation. The nuclei involved in feeding regulation are the arcuate nucleus (AN), the ventromedial nucleus (VMH), the dorsomedial nucleus (DMD), the PVN, and the lateral hypothalamic area (LHA). HFD, in addition to its effect on the HPA axis, has been shown to modulate neurotransmitter concentrations which play a role in regulating feeding behavior [129]. NE including other monoamines dopamine (DA) and serotonin (5-hydroxytryptamine; 5HT), in addition to their metabolites 3,4-Dihydroxyphenylacetic acid (DOPAC) and 5-Hydroxyindoleacetic acid (5-HIAA) respectively, are of interest as they play a major role in feeding regulation. HFD has also been shown to cause a low-grade inflammatory state within the CNS, potentially disrupting neurotransmitter signaling within the brain potentially contributing to the development of obesity [129].

The interaction between these two systems, the HPA axis and the feeding centers, in response to prenatal surgical stress and HFD are the focus of this study. We hypothesize that maternal surgical stress and HFD feeding will increase monoamine levels in nuclei involved in stress axis regulation leading to an exacerbated stress response while

simultaneously suppressing monoamine levels in feeding related nuclei leading to reduced satiety. The combination of the two effects results in increased food intake, altered metabolism and obesity.

4.3 MATERIALS AND METHODS

Animals and treatment

Animal Care. Animals were handled and treated the same as previously described in 3.3 materials and methods.

Offspring Care and Dietary Manipulation. Offspring were handled and treated the same as previously described in 3.3 materials and methods. A schematic of the experimental protocol is provided in Fig 4.1.

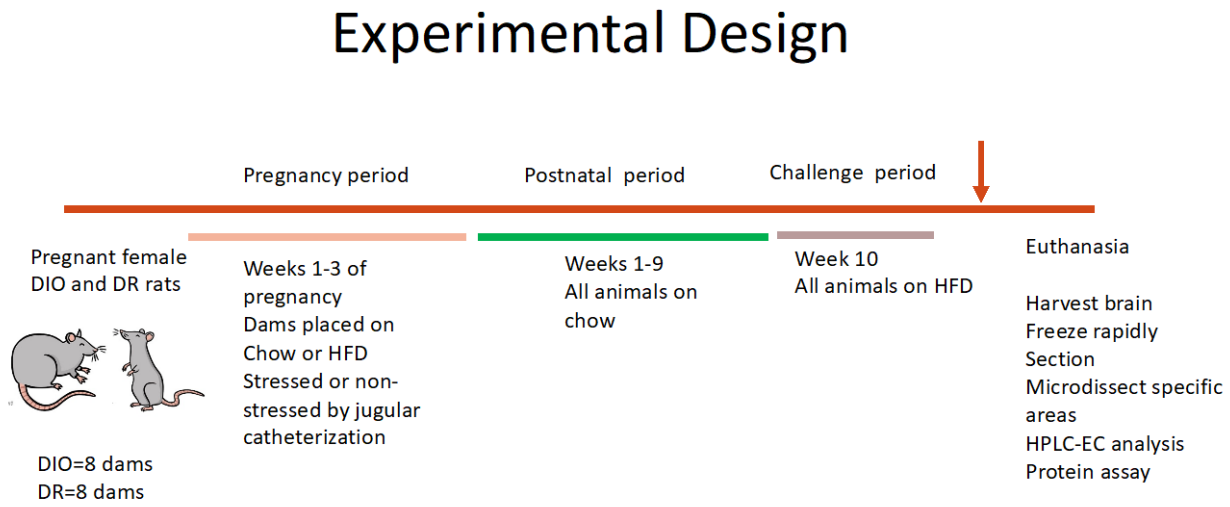

Figure 4.1: Experimental Design.

Pregnant DIO and DR rats (n=8) were evenly divided (n=4) into prenatal stress and non-stress (control) groups. The prenatal stress group underwent jugular catheterization once a week for 3 weeks. They were fed either chow or HFD for the duration of the pregnancy. After birth, the male offspring from each prenatal programming group (n=16) were weaned off their mothers at 3 weeks of age and fed chow diet (CD) for 6 weeks. After 9 weeks of age, the offspring were fed HFD for 1 week. After 1 week of postnatal treatment, the offspring were euthanized by decapitation and the brains were collected and stored at -80°C until processing.

These methods were done in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Sectioning and Microdissection of the Brain

After euthanasia, the brains were harvested, and immediately frozen in dry ice and stored at -80°C until they were cryosectioned at a thickness of 300 µm. The sectioning was performed with a cryostat maintained at -10°C. After sectioning, 9 specific regions of the brain: cortex (CX), bed nucleus of the stria terminalis (BNST), paraventricular nucleus (PVN), central amygdala (CeA), lateral hypothalamic area (LH), arcuate nucleus (AN), ventromedial nucleus (VMH), dorsomedial nucleus (DMD), and hippocampus (HI) were micro dissected using Palkovits' micro-punch (500 µm) method [155]. Each region was micro-punched bilaterally on a cold stage (FTS Systems Inc., Stoney Ridge, NY) maintained at -10°C. The samples were then homogenized in 150 µl of 0.5 M $HClO_4$ and

two aliquots of 5µL each were saved for protein assay analysis. A BCA protein assay was used to measure the amount of protein in the homogenized solution to normalize the neurotransmitter concentrations between samples. The rest of the sample was centrifuged at 12000 x g and stored at -70°C until analysis by high performance liquid chromatography with electrochemical detection (HPLC-EC).

HPLC-EC

HPLC-EC was conducted to measure the concentrations of neurotransmitters: NE, DA, 5HT, and their metabolites in the regions mentioned above. 50 µl of the homogenate was mixed with 25 µl of the internal standard (0.05M dihydroxybenzylamine) before being injected into the HPLC-EC machine. NE, DOPAC, DA, 5HIAA, and 5HT concentrations in the CX, BNST, PVN, CeA, LH, AN, VMH, DMD, and HI were measured using HPLC-EC as previously described [156].

The HPLC-EC system comprised of a 5-µm ODS reverse phase C-18 column (Phenomenex, Torrance, CA), a SIL-20AC autoinjector, a CTO-20AC column oven (Shimadzu, Columbia, MD) maintained at 37°C and a LC-4C detector (Bioanalytical Systems, West Lafayette, IN). The flow rate of the mobile phase was maintained at 1.8 ml/min using a LC-20AD pump (Shimadzu, Columbia, MD). Chromatograms were analyzed for neurotransmitter concentrations using the Class VP software v 7.2 (Shimadzu, Columbia, MD). Neurotransmitter concentrations in tissue samples were expressed as pg/µg of protein.

Statistical analysis

Differences in neurotransmitter levels between genotypes, stress conditions and diet were analyzed using GraphPad Prism software version 9.0.0 (GraphPad Inc., San

Diego, CA). Neurotransmitter data were compared using 3-way ANOVA followed by Fisher's LSD post hoc test. Sidak's multiple comparison post-hoc test was used to perform multiple comparisons holding either genotype, stress, or diet as a single factor for comparison at any one time. A p value less than 0.05 was considered significant but differences less than $p<0.01$ are reported. Data are expressed as Mean ± SEM.

4.4 RESULTS

Effects of Prenatal Stress and maternal HFD on monoamine neurotransmitter levels in hypothalamic nuclei related to stress in male and female DIO and DR offspring

NE levels in the PVN, AN, and cortex (CX; control region) were measured by the analysis of HPLC-EC data. Significant effects were observed in these nuclei at differing levels depending on the sex of the animals which can be observed in figure 4.2. Males exhibited higher levels of NE in the PVN and in the AN overall. NE was significantly increased in DIO males that were born of stressed and HFD fed dams compared to males born to DIO non-stressed HFD and DIO stressed CD fed groups. This indicates there is a significant diet and stress effect on NE in the PVN in males. NE was significantly decreased in DR males that were born to stressed and HFD fed dams compared to DIO males born to stressed HFD fed dams as well. This indicates a significant genotype effect on NE in the PVN. Females exhibited the complete opposite effects in the PVN as DR females born to stressed and HFD fed dams had the highest levels of NE compared to the other DR groups. This indicates a diet and stress effect on NE in the PVN in females. The effect on NE in the AN was almost identical in males with significant differences between the same groups. The effect on NE in the AN in females was more normal except that NE was significantly decreased in DIO animals born to stressed and HFD fed dams.

No significant difference was observed in the CX of the males, but a significant difference was observed in the CX in females. While stress increased NE levels in chow-fed females, feeding dams HFD completely blocked this increase in the AN. A genotype effect was also observed between HFD females with DR females having higher NE levels in the cortex compared to DIO females.

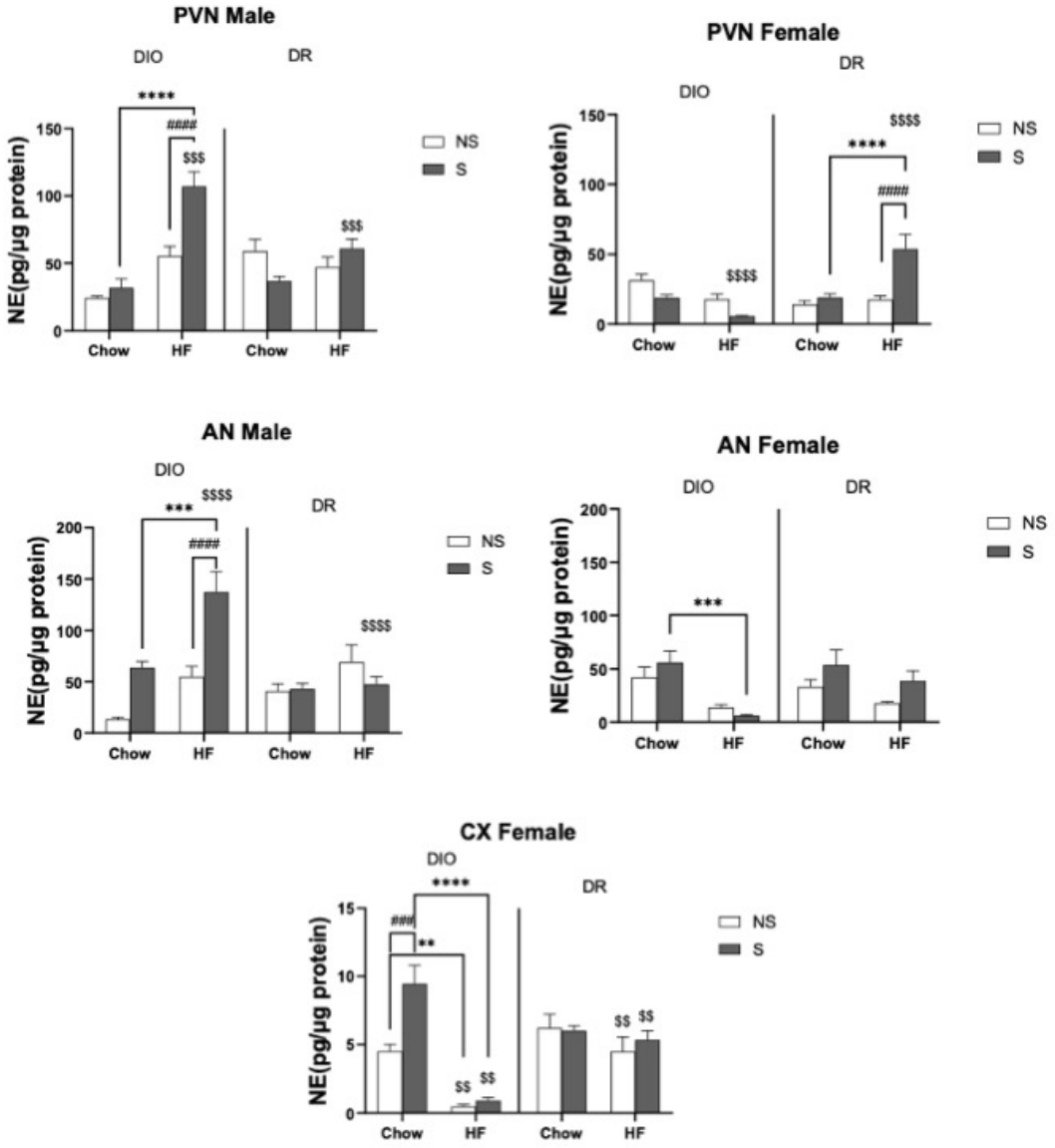

Figure 4.2: NE levels in control and hypothalamic stress-related nuclei in DIO/DR offspring after prenatal surgical stress and maternal HFD consumption upon challenge with HFD in adulthood.

NE levels in the control region (CX) and hypothalamic regions related to stress (PVN and AN) are depicted here. Neurotransmitter data were compared using 3-way ANOVA followed by Fisher's LSD post hoc test. Sidak's multiple comparison post-hoc test was used to perform multiple comparisons holding either genotype, stress, or diet as a single factor for comparison at any one time. CD-chow diet; HFD-high fat diet; S-prenatal stress; NS-non-stressed; DIO-diet-induced obese and DR-dietary resistant. Prenatal stress programming combined with maternal HFD consumption produced significant effects on NE levels. Graphs represent mean ± SEM from 6-9 offspring per treatment group. Asterisk (*), dollar sign ($), and pound sign (#) represents a significant difference between diet, genotype, or stress/non-stress respectively. ** indicates (p <0.01); *** (p<0.001); ****(p<0.0001).

Significant effects were observed in DA levels in the PVN and CX due to prenatal stress, diet, and genotype and these are depicted in figure 4.3. DA levels in the PVN were significantly increased in DIO non-stressed HFD males compared to chow fed males. DA was also significantly increased in DIO stressed CD and HFD females compared to non-stressed females. There was a significant genotype effect in the PVN, with DA levels being significantly reduced in DR stressed HFD females compared to stressed DIO females on the same diet. DA levels in the CX were also significantly increased in DR non-

stressed HFD males compared to the rest of the groups. A significant difference was also observed in females in the CX between chow fed DIO stressed vs non-stressed animals.

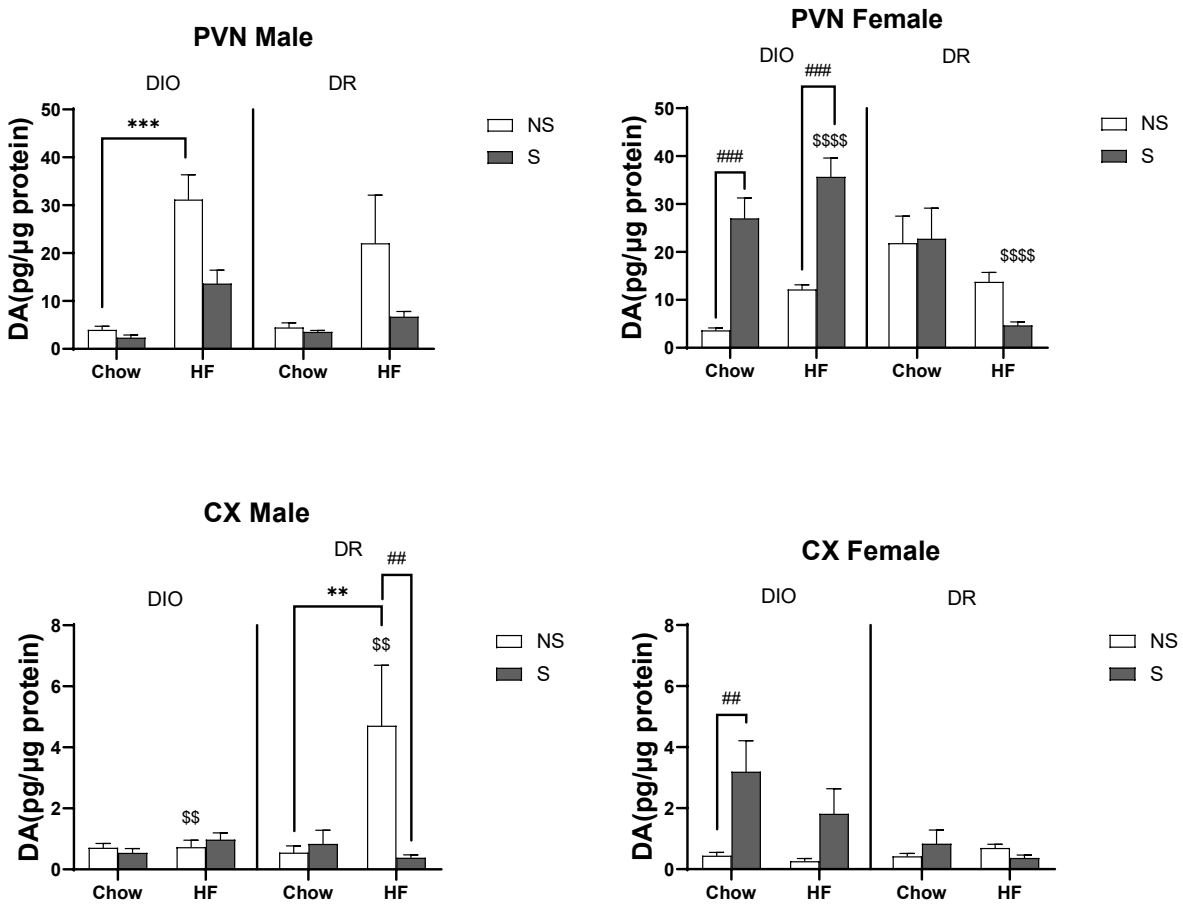

Figure 4.3: DA levels in control and hypothalamic stress-related nuclei in DIO/DR male/female rats after prenatal stress/non-stress and maternal CD/HFD consumption.

DA levels in the control region (CX) and hypothalamic regions related to stress (PVN and AN) are depicted here. Neurotransmitter data were compared using 3-way ANOVA followed by Fisher's LSD post hoc test. Šídák's multiple comparison post-hoc test was

used to perform multiple comparisons holding either genotype, stress, or diet as a single factor for comparison at any one time. CD-chow diet; HFD-high fat diet; S-prenatal stress; NS-non-stressed; DIO-diet-induced obese and DR-dietary resistant. Prenatal stress programming combined with maternal HFD consumption produced significant effects on DA levels. Graphs represent mean ± SEM from 6-9 offspring per treatment group. Asterisk (*), dollar sign ($), and pound sign (#) represents a significant difference between diet, genotype, or stress/non-stress respectively. ** indicates (p <0.01); *** (p<0.001); ****(p<0.0001).

Significant effects were observed in 5HT levels in the PVN, AN, and cortex (CX; control region) at differing levels depending on the sex of the animals which can be observed in figure 4.4. Significant differences in levels of 5HT were only observed in the PVN in males. 5HT was significantly increased in DIO non-stressed HFD males compared to DIO non-stressed CD and DR non-stressed HFD. This indicates a significant diet and genotype effect on 5HT in the male PVN. 5HT levels were significantly increased in the AN of DIO stressed HFD males compared to DIO stressed CD, DIO non-stressed HFD, and DR stressed HFD males indicating a diet, stress, and genotype effect respectively. 5HT levels were significantly increased in the AN of DIO non-stressed HFD females compared to DIO stressed HFD and DR non-stressed HFD indicating a significant stress and genotype effect respectively. There were no significant differences in 5HT levels in the CX of the males, but there were significantly increased levels of 5HT in the CX of DIO stressed CD females compared to DIO stressed HFD females indicating a significant diet effect.

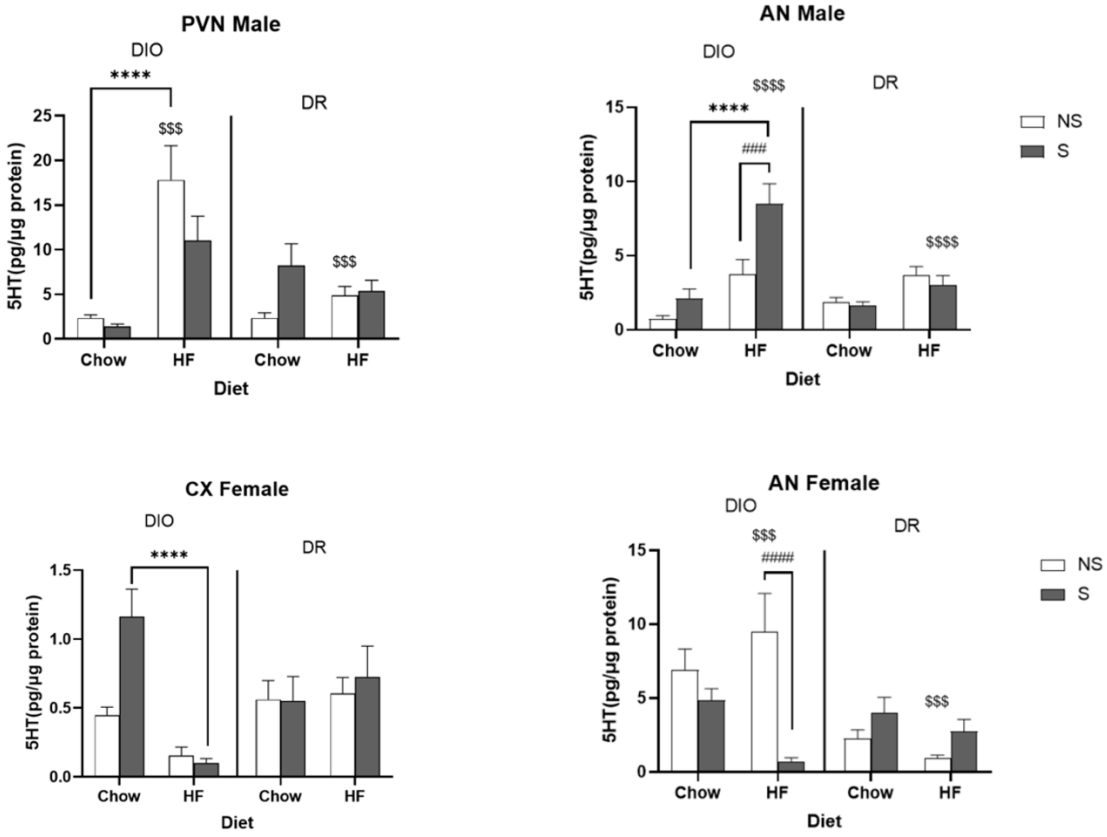

Figure 4.4: 5HT levels in control and hypothalamic stress-related nuclei in DIO/DR male/female rats after prenatal stress/non-stress and maternal CD/HFD consumption upon challenge with HFD in adulthood.

5HT levels in the control region (CX) and hypothalamic regions related to stress (PVN and AN) are depicted here. Neurotransmitter data were compared using 3-way ANOVA followed by Fisher's LSD post hoc test. Šídák's multiple comparison post-hoc test was used to perform multiple comparisons holding either genotype, stress or diet as a single factor for comparison at any one time. CD-chow diet; HFD-high fat diet; S-prenatal stress; NS-non-stressed; DIO-diet-induced obese and DR-dietary resistant. Prenatal stress

programming combined with maternal HFD consumption produced significant effects on 5HT levels. Graphs represent mean ± SEM from 6-9 offspring per treatment group. Asterisk (*), dollar sign ($), and pound sign (#) represents a significant difference between diet, genotype, or stress/non-stress respectively. ** indicates (p <0.01); *** (p<0.001); ****(p<0.0001).

Effects of Prenatal Stress and maternal HFD consumption on monoamine neurotransmitter levels in hypothalamic nuclei related to feeding regulation in DIO and DR offspring that were challenged with 1 week of HFD

NE levels in the LH, VMH, and DMD were measured by the analysis of HPLC-EC data. Significant effects were observed in these nuclei at differing levels depending on the sex of the animals which can be observed in figure 4.5. In the LH, NE was significantly decreased in DIO stressed HFD females compared to DIO stressed CD females indicating a significant diet effect. NE was also significantly increased in the LH of DR non-stressed HFD females compared to DIO non-stressed HFD females indicating a significant genotype effect. There were no significant differences in NE levels in the LH of males. In the VMH, NE was significantly decreased in DR non-stressed HFD males compared to DIO non-stressed HFD males indicating a significant genotype effect. In the VMH of females, NE levels were much lower than males. NE levels were significantly higher in DIO stressed CD females compared to DIO non-stressed CD and DIO stressed HFD females indicated a significant stress and diet effect. NE levels were also significantly higher in the VMH of DR stressed HFD females compared to DR non-stressed HFD and DIO stressed HFD females indicating another significant stress effect as well as

a genotype effect. In the DMD, there were no significant differences in levels of NE in males, but in females NE levels were significantly higher in DIO stressed CD compared to DIO stressed HFD indicating a significant diet effect.

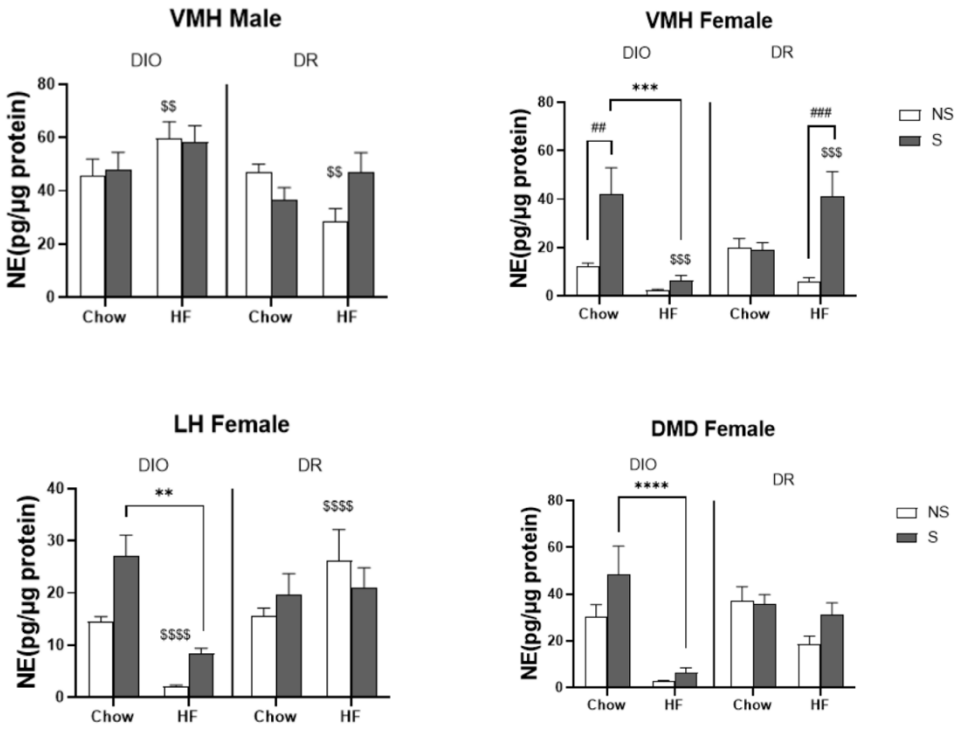

Figure 4.5: NE levels in hypothalamic feeding regulation nuclei in DIO/DR male/female rats after prenatal stress/non-stress and postnatal CD/HFD consumption.

NE levels in the hypothalamic regions related to feeding are depicted here. Neurotransmitter data were compared using 3-way ANOVA followed by Fisher's LSD post hoc test. Šídák's multiple comparison post-hoc test was used to perform multiple comparisons holding either genotype, stress, or diet as a single factor for comparison at any one time.

CD-chow diet; HFD-high fat diet; S-prenatal stress; NS-non-stressed; DIO-diet-induced obese and DR-dietary resistant. Prenatal stress programming combined with maternal HFD consumption produced significant effects on NE levels. Graphs represent mean ± SEM from 6-9 offspring per treatment group. Asterisk (*), dollar sign ($), and pound sign (#) represents a significant difference between diet, genotype, or stress/non-stress respectively. ** indicates (p <0.01); *** (p<0.001); ****(p<0.0001).

DA levels in the LH, VMH, and DMD were measured by the analysis of HPLC-EC data. Significant effects were observed in these nuclei at differing levels depending on the sex of the animals which can be observed in figure 4.6. In the LH, DA levels were very similar between males and females except for the DIO stressed HFD male group. This group had significantly higher levels of DA in the LH compared to DIO stressed CD, DIO non-stressed HFD, and DR stressed HFD indicating a significant diet, stress, and genotype effect. In the LH of the females, DA was only significantly increased in DIO stressed HFD females compared to DIO non-stressed HFD females indicating a significant stress effect. In the VMH, there were no significant differences in the level of DA in males. In the VMH of females, DA was significantly increased in DIO stressed CD females compared to both DIO non-stressed CD females and DR stressed CD females indicating a significant stress and genotype effect. In the DMD, there were no significant differences in the level of DA in males. In the DMD of females, DA was significantly increased in DIO stressed CD females compared to DIO non-stressed CD, DIO stressed HFD, and DR stressed CD females indicating a significant stress, diet, and genotype effect.

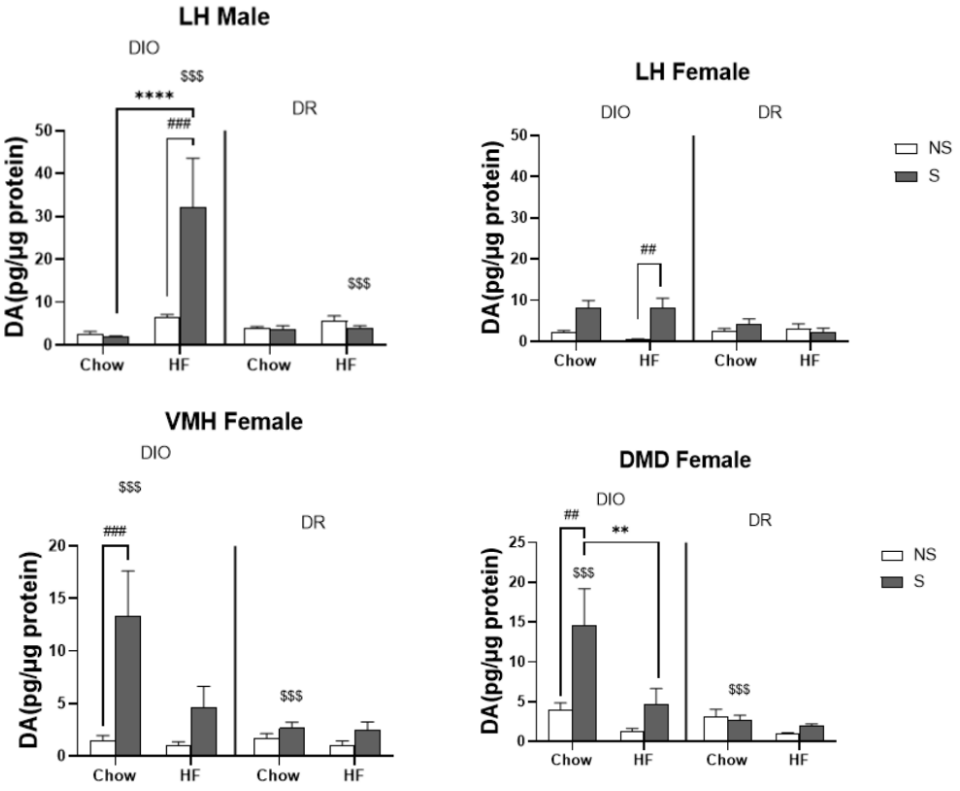

Figure 4.6: DA levels in hypothalamic feeding regulation nuclei in DIO/DR male/female rats after prenatal stress/non-stress and postnatal CD/HFD consumption.

DA levels in the hypothalamic regions involved in the regulation of feeding are depicted here. Neurotransmitter data were compared using 3-way ANOVA followed by Fisher's LSD post hoc test. Šídák's multiple comparison post-hoc test was used to perform multiple comparisons holding either genotype, stress, or diet as a single factor for comparison at any one time. CD-chow diet; HFD-high fat diet; S-prenatal stress; NS-non-stressed; DIO-diet-induced obese and DR-dietary resistant. Prenatal stress programming combined with maternal HFD consumption produced significant effects on DA levels. Graphs

represent mean ± SEM from 6-9 offspring per treatment group. Asterisk (*), dollar sign ($), and pound sign (#) represents a significant difference between diet, genotype, or stress/non-stress respectively. ** indicates (p <0.01); *** (p<0.001); ****(p<0.0001).

5HT levels in the LH, VMH, and DMD were measured by HPLC-EC. Significant effects were observed in these nuclei at differing levels depending on the sex of the animals which can be observed in figure 4.7. In the LH, VMH, and DMD of males, 5HT was not significantly different between any groups. In the LH of females, 5HT was significantly increased in DR non-stressed HFD animals compared to DIO non-stressed HFD indicating a significant genotype effect. In the VMH of females, 5HT was significantly increased in the DIO stressed CD animals compared to DIO stressed HFD indicating a significant diet effect. In the DMD of females, 5HT was significantly increased in DIO non-stressed CD animals compared to DIO non-stressed HFD indicating a significant diet effect in the DMD as well.

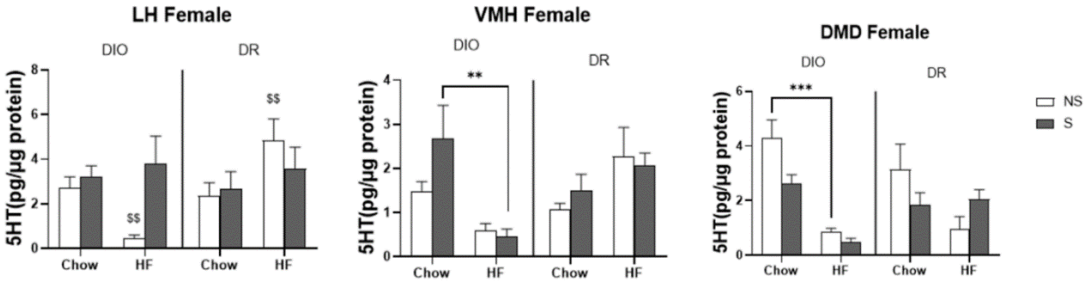

Figure 4.7: 5HT levels in hypothalamic feeding regulation nuclei in DIO/DR male/female rats after prenatal stress/non-stress and postnatal CD/HFD consumption.

5HT levels in the hypothalamic regions related to feeding are depicted below. Neurotransmitter data were compared using 3-way ANOVA followed by Fisher's LSD post hoc test. Šídák's multiple comparison post-hoc test was used to perform multiple comparisons holding either genotype, stress, or diet as a single factor for comparison at any one time. CD-chow diet; HFD-high fat diet; S-prenatal stress; NS-non-stressed; DIO-diet-induced obese and DR-dietary resistant. Prenatal stress programming combined with maternal HFD consumption produced significant effects on 5HT levels. Graphs represent mean ± SEM from 6-9 offspring per treatment group. Asterisk (*), dollar sign ($), and pound sign (#) represents a significant difference between diet, genotype, or stress/non-stress respectively. ** indicates (p <0.01); *** (p<0.001); ****(p<0.0001).

4.5 DISCUSSION

Over the past few decades, obesity has become a major public health challenge around the world [157, 158]. One of the greatest economic impacts of obesity has been the costs related to associated diseases and conditions such as metabolic syndrome, arthritis, cancer, sleep disorders, type 2 diabetes, and heart disease [159, 160]. These have led to marked increases in healthcare related expenditures while simultaneously decreasing productivity due to lost days at work [161, 162]. Therefore, understanding the mechanisms that promote the development of obesity may lead to new, more effective interventions or therapies.

As previously mentioned, stress and HFD play a major role in feeding behavior and promoting hyperphagia leading to the development of obesity [163, 164]. Prenatal programming has also been shown to promote the development of diseases in the adult offspring [165, 166]. Therefore, we theorized that the combination of prenatal stress programming and maternal HFD consumption would predispose and promote the development of obesity when offspring are challenged with HFD. We investigated monoamine neurotransmitters levels in specific nuclei of the hypothalamus that are involved in feeding to determine if their modulation might be involved in the development of obesity.

The nuclei that are involved in regulating feeding behavior are the PVN, AN, VMH, DMD, and LHA [167]. The monoamine neurotransmitters being investigated are NE, DA, and 5HT and their role on feeding regulation are depicted in figure 4.8. NE is the stress hormone and plays a major role in the HPA axis [168]. NE also plays a role in stimulating feeding behavior when its levels increase in the PVN [169]. DA is distributed throughout the brain and plays a role in many functions including the reward system and feeding regulation [170]. When DA levels are low in the AN, VMH, LH, and DMD appetite levels are increased which induces feeding. When DA levels are high appetite levels are decreased which prevents feeding [102]. 5HT has been investigated thoroughly because of its role in treating neurological disorders such as anxiety and depression. 5HT in the PVN plays a stimulatory role on sympathetic drive which increases energy expenditure, most probably by promoting thermogenesis by brown adipose tissue [170]. On the other hand, 5HT levels in the PVN are also known to suppress the stimulatory effects of ghrelin on feeding behavior and prevents utilization of carbohydrates for energy [171].

By investigating the changes in the levels of these neurotransmitters in response to prenatal stress programming and maternal HFD, we may be able to elucidate the underlying mechanisms involved. The increased levels of NE in the PVN of DIO stressed and HFD fed males is appropriate as stress axis activity is increased in these animals. A similar increase in NE levels in the PVN of DIO rats after HFD feeding was reported earlier [154]. The opposite is true for the PVN in the females where NE barely increased in the DIO animals but increased significantly in DR animals when their moms were placed on a HF diet. These effects may support increased stress axis activity in HFD fed DIO males and DR females. The sex related differences in stress axis activity in response to HFD between these two genotypes is interesting and needs to be investigated further. In the AN, NE levels increased in HF fed DIO males like the PVN. The AN contains both orexigenic NPY/AgRP neurons and anorexigenic POMC neurons. Increase in NE levels could stimulate the orexigenic neurons through excitatory alpha 1a and beta-adrenergic receptors while inhibiting POMC neurons through alpha 2a adrenergic receptors [172]. Moreover, there are peptidergic neuronal connections between the AN and the PVN [173] so it is possible that the changes induced by prenatal stress and the HFD in the PVN could directly influence the changes in the AN. The cortex was used as a control region, but interestingly, NE in the CX showed no statistical significance in males, but in females, NE was significantly low in DIO non-stressed and stressed HFD fed animals. NE in the cortex has been implicated in working memory and attention [174]. The levels of NE in the CX were almost the opposite to that in the PVN and AN in males. While the increases in NE levels in the PVN could directly be implicated in stress and increased feeding in DIO animals, the reduced levels of NE in the CX could contribute to reduced

attention, learning and sluggishness. These results also indicate that DIO rats are more susceptible to the modulations caused by the prenatal stress and HFD treatment.

NE levels were elevated in the VMH of female DIO rats in response to prenatal stress. However, this increase was abolished when DIO animals were placed on HFD. In contrast, DR animals placed on HFD had marked elevations in NE in the VMH particularly when they had been subjected to prenatal stress. NE levels in the VMH are known to be important for feeding behavior since chronic infusion of NE into the VMH promotes obesity [175]. This was accompanied by increased day time feeding, arrhythmic feeding patterns and excessive weight gain. Triglyceride turnover in the brown adipose tissue was greatly reduced, but there was a marked elevation in plasma insulin levels [175]. It might be worthwhile to study the circulating levels of insulin in DIO chow and DR HFD fed females.

In contrast to the VMH, there were modest changes in NE levels in the DMD and LH of female rats. While chow fed DIO rats that were prenatally stressed, had higher levels of NE in the DMD and LH, HFD feeding completely suppressed this increase. The levels of NE in the LH are known to influence feeding in several ways. Injection of NE into the lateral hypothalamus is known to stimulate feeding [176]. Moreover, feeding induced by electrical stimulation of the LH can be abrogated by administration of agents that deplete NE [177] indicating a stimulatory role for NE levels in the LH in feeding behavior. On the other hand, LH neurons that regulate feeding appear to be controlled not only by neural pathways but also by circulating factors such as insulin, glucagon and free fatty acids [178]. In the present study, NE levels were unaffected in the LH of males but

there was a modest reduction in female DIO rats subjected to prenatal stress and HFD. This could contribute to reduced feeding in these animals.

As mentioned previously, DA is present throughout the brain. In the PVN, DA has recently been shown to stimulate food intake by acting through D1 receptors [179]. There is also some evidence to suggest that DA in the PVN may counter stress axis activation [180]. Results from the present study clearly show that DA levels are elevated in stressed DIO females (regardless of diet). This could be a modulation that has occurred due to the prenatal stress programming. The DR females in contrast, have reduced levels of DA in the PVN indicating that this could be a genetic adaption favoring reduced feeding. The levels of DA in the CX even show this trend of increased DA in stressed DIO animals fed a CD. The DA levels in the PVN and CX of males are as would be expected: increased levels of DA in animals fed HFD, but lower in stressed animals. Therefore, there could be a sex difference that predisposes the females to increased DA signaling in response to stress. DA levels in the cortex have been implicated in cognitive control [181] and anxiety like behavior [182]. It is not clear if the increased dopamine in the cortex observed in certain groups has any implication in these behaviors. This warrants further study.

Female and male DIO offspring have increased levels of DA in the LH when subjected to prenatal stress. However, this increase in DA levels were evident in males only when they were challenged with HFD. The role of DA in the LH is associated with antinociception. The implication of this finding related to the present study is not clear. The LH, VMH, and DMD also exhibited increased levels of DA in stressed animals compared to non-stressed animals. Again, this could be a result of epigenetic changes induced by

prenatal stress. DA is believed to act through D2 receptors in the VMH to increase feeding [183]. The increased levels of DA in the VMH of DIO stressed and CD fed females most probably promotes feeding in these animals. Increased levels of DA in the LH of DIO males and females fed HFD could be appropriate as DA in the LH has been shown to act through D1 and D2 receptors and different neuropeptides to inhibit feeding [184].

High fat feeding increases the expression of 5HT(1B) receptor expression in the AN and LH of DIO rats. 5HT transporters increase with high fat feeding in the PVN, LH, and VMH in DIO rats. These changes are associated with reduced levels of 5HT in these areas as a consequence of HFD which could contribute to increased appetite [185]. On the contrary, elevated 5HT levels in the PVN, VMH, DMD, and LH would reduce appetite and increase energy expenditure [107]. Altogether, 5HT could contribute to the homeostatic circuitry to match energy intake to energy expenditure or to the hedonic circuitry that is associated with reward and motivational behavior related to food consumption [186]. Increased levels of 5HT were observed in the PVN of DIO HFD fed males which could lead to suppression of feeding. In the AN, 5HT levels were found at higher levels in DIO stressed HFD males and DIO non-stressed HFD females. 5HT innervates NPY neurons in the AN [187] which could be a mechanism by which 5HT stimulates feeding. 5HT levels were significantly low in the LH, VMH, and DMD of females with no observed changes in males. The significantly low levels in the females could lead to increased appetite and feeding as explained above.

In conclusion, our study reveals that prenatal stress in combination with maternal HFD exposure modulates monoamine neurotransmitter levels within hypothalamic nuclei that regulate feeding behavior when offspring are challenged with HFD in adulthood.

Further studies are necessary to understand the exact mechanisms involved as this is an intricately interconnected system as depicted in Fig 4.8.

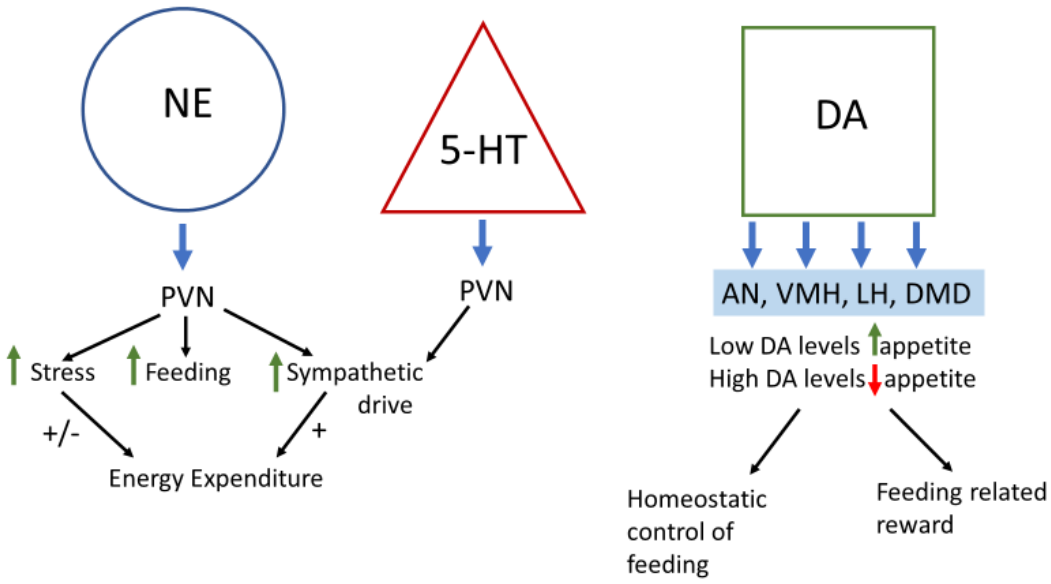

Figure 4.8: Monoamine neurotransmitter regulation of feeding.

An illustrative representation of the interaction between the three monoamine neurotransmitters involved in the regulation of feeding. They are norepinephrine (NE), serotonin (5HT), and dopamine (DA). The nuclei in which they act and their role on feeding behavior/energy homeostasis is depicted. PVN = paraventricular nucleus, AN = arcuate nucleus, VMH = ventromedial nucleus, LH = lateral hypothalamic area, and DMD = dorsomedial nucleus.

CHAPTER 5

CONCLUSIONS AND SIGNIFICANCE

PLX3397 was shown to have an effect in suppressing the inflammatory state within the hypothalamic feeding centers. Changes in metabolism and monoamine neurotransmitters in hypothalamic feeding centers that favor the development of obesity were observed in response to the programming induced by prenatal stress and HFD feeding. Multiple effects were observed between the groups whether they were diet, stress, genotype, or sex. Additional investigations building upon this research will aid in the understanding of the multiple factors that promote obesity and possibly produce novel strategies or therapies to address this condition.

The regulation of feeding behavior is a complex phenomenon in which multiple body systems are involved. The liver, pancreas, adipose tissue, gut, and muscle give rise to signals that are integrated by the brain [167, 188]. Several regions of the brain and specific hypothalamic nuclei play a major role in maintaining energy homeostasis [189]. Within the brain, several neurotransmitter systems provide interweaving connections that help to finely tune responses that are appropriate for the survival and health of the organism [190-195]. For the most part, energy homeostasis is well controlled and delicately balanced [196]. However, several environmental factors can easily disrupt this balance. One of these major environmental factors is the consumption of a high fat diet (HFD). HFD has been shown to promote increased food consumption mainly because it is highly palatable and stimulates reward centers in the brain [197, 198]. It also induces

'hyperphagia': it's hard to resist a highly palatable meal and frequently this results in overeating which is one of the major contributors to the development of obesity. It is to be noted that consumption of HFD for a short duration of time will not cause obesity but will lead to changes in glucose homeostasis and induce metabolic changes that gathers speed and destructive power as it goes downhill [199, 200]. In addition to the fact that HFD causes hyperphagia, it also induces an inflammatory state within the central nervous system [201, 202]. This inflammatory state may also play a role in the development of obesity, but it has been theorized that anti-inflammatory drugs may be a therapy able to target this mechanism [203]. Other factors contributing to the development of obesity that are not related to the environment include genetics [204, 205], epigenetics [206], nutritional status during gestation [207, 208], maternal obesity [209], prenatal stress [210], and the interaction between some or all of these factors [211, 212]. Studies have been conducted for the past 30 or so years to investigate maternal prenatal programming factors such as stress and nutrition that may predispose or 'program' the offspring to the development of diseases later into adulthood. This phenomenon is known as the developmental origins of health and disease or the "Barker Hypothesis" and was coined by David Barker in 1986 [75, 213]. Stress has been considered an environmental factor promoting the development of obesity, but stress activates what is called the hypothalamo-pituitary adrenal axis or the 'stress axis.' This connection of stress to the hypothalamus is of interest as the hypothalamus is the region of the brain that maintains energy homeostasis/feeding regulation [214]. Feeding regulation is a complex mechanism with multiple factors including specific hypothalamic nuclei involved in regulating feeding, neuropeptides, neurotransmitters, and signals from the gut, brain axis. The hypothalamic nuclei

involved, that were also of interest in the inflammatory state, are the paraventricular nucleus (PVN), arcuate nucleus (AN), ventromedial nucleus (VMH), dorsomedial nucleus (DMD), and lateral hypothalamic area (LH). As these regions were already of interest based on the concern for HFD and the promotion of an inflammatory state, the neurotransmitters involved within these regions that also help regulate feeding behavior have become of major interest [215]. The monoamine neurotransmitters norepinephrine (NE), dopamine (DA), and serotonin (5HT) are the three major neurotransmitters involved and have been studied for many years [216-219]. The idea of how prenatal stress and postnatal high fat diet might affect the body's metabolism as well as modulate neurotransmitter levels within the brain affecting feeding regulation is of major importance for the better understanding of how obesity is developed. These results will then allow us to draw conclusions on how the inflammatory state may also be playing a role in this mechanism of obesity development.

5.1 Obesity Development and Prevention

Although studies have shown that HFD causes an inflammatory state within the CNS and the development of obesity, specific details on how anti-inflammatory drugs like PLX3397 might reduce inflammation and positively affect the regions that control feeding regulation have yet to be revealed. PLX3397 is an inhibitor of Colony stimulating factor 1 receptor and has been frequently used to deplete microglia in the brain [220]. In this study, this was evaluated using rats in multiple treatment groups and in males and females to investigate if the drug was effective in more than one diet or not and to delineate any sex differences. Our results indicate that PLX3397 did suppress neuroinflammation

in HFD treated male and female rats which was indicated by less microglia activation in the PVN, AN, DMD, and LH.

Prenatal stress has been linked to the development of obesity in later life [210]. As a next step we investigated the effect of prenatal stress on the development of obesity in genetically obesity prone (diet-induced obese) and resistant (dietary resistant) rat models. Several paradigms of prenatal stress are used in the literature including restraint stress, dietary restriction etc. We used surgical blood collection as a stress paradigm for our studies. This method was used to detect changes in biomarker levels in maternal circulation in DIO and DR rats that led to changes in offspring phenotypes. Interestingly, the short surgical procedure that was done once a week produced a major increase in appetite in offspring that were born of these dams. We followed the prenatal stress with exposure to a high fat or chow diet for 1 week in adulthood. Food intake, body weight, corticosterone levels, fat levels, and leptin were collected and analyzed. We tested the hypothesis that prenatal stress in combination with maternal HFD consumption may cause increased activation of the stress axis as corticosterone levels were significantly altered. Differences were observed between males and females indicating that further investigation is required to fully understand the mechanisms involved. Prenatal stress and maternal HFD consumption increased food consumption as expected when offspring were challenged with HFD. Prenatal stress and maternal HFD consumption led to increased fat accumulation. Increased fat accumulation was more significant in DIO animals as expected, but also in males. Stress played a major role in fat accumulation as the prenatally stressed offspring developed increased levels of fat. Leptin levels were lower than expected in HFD fed animals as leptin levels should be increased inhibiting hunger. Although this

interaction may not have been long enough for leptin levels to increase as it is a more long-term inhibitor of food intake. Lastly body weight did increase, but not significantly. Again, the study may not have been long enough for major weight gain to occur, but it did appear to be on the rise with fat accumulation and food intake increasing for many of the groups.

To further explore the underlying mechanisms for prenatal stress-induced obesity in adulthood, we hypothesized that alterations in neurotransmitter levels in specific hypothalamic nuclei may drive the increase in food intake and reduction in energy expenditure leading to obesity in the offspring. Many significant changes were observed between the groups in the DIO vs DR, non-stressed vs stress, and CD vs HFD fed rats. The first major modulation that was observed was in NE. Higher levels of NE were observed in the PVN of DIO stressed and HFD fed males which is what was to be expected. Although, low levels of NE were found in the PVN of all the female DIO groups. This is significant as NE should at least be elevated in the stressed group, but it was not in the PVN. This was the case for multiple nuclei between males and females. The conclusion was drawn that males and females may differ in where stress and HFD modulate NE to promote increased food consumption promoting obesity and this will need to be investigated in more detail. Another potential modulation in response to this experimental design was observed in the levels of DA in multiple groups. DA plays a role in multiple systems, but what stood out was how DA levels were high in regions like the PVN, LH, VMH, and DMD in DIO stressed females while DA levels were low in the regions for the males of the same group. The opposite was the case in the DR stressed females as well. Therefore, there could be a genetic adaptation in DR females that prevented high DA levels. There

could also be a mechanism between the sexes that modulates the levels of DA in females vs males. Lastly, 5HT levels were significantly low in the LH, VMH, and DMD of females. It was concluded that there may be a mechanism involved that is inhibiting the release of 5HT which would counteract 5HT's inhibitory effect on feeding regulation and potentially promote the development of obesity.

5.2 Study limitations

The DIO/DR rat model is unique and must be maintained in a rat colony that requires significant resources. The model itself closely resembles human obesity because it is polygenic, and the pairing of DIO and DR models can help to delineate and dissect the possible factors that are involved in the development of obesity. The duration of high fat diet exposure in this study was for 1 week. Although earlier studies have found dramatically less changes in neurotransmitter systems with longer durations of HFD exposure [221, 222], we could use other paradigms to increase energy intake such as high sucrose or a different form of high energy diet to determine if neurotransmitter changes are altered by the energy source.

5.3 Future Directions

There are several pathways that could be explored: The role of inflammatory mediators and oxidative stress in altering neurotransmitter levels and their interaction with neuropeptides and other hormones to develop obesity is one pathway. We can investigate the effects of prenatal stress on the gut microbiome, gut hormones and how they regulate feeding. We can study the role of insulin sensitivity, insulin resistance, and adipocyte-based alterations in response to prenatal stress and how they contribute to obesity. Investigating the mechanisms involved in the sex specific effects as previously mentioned. For

the sex specific differences, comparing data at different periods of the estrous cycle may play a role in females. Investigating the components of the hypothalamo-pituitary-gonadal (HPG) axis in both males and females could be another target when investigating the role of sex hormones in response to this experimental design. Lastly, investigating the inflammation and inflammatory pathways in this mechanism as well to compare Sprague-Dawley to the DIO/DR animal model.

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