EFFECTS OF MATERNAL HIGH FAT DIET ON HYPOTHALAMUS VASCULARITE OF THEIR OFFSPRING

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Approval of The Thesis

EFFET OF MATERNAL HIGH FAT DIET ON HYPOTHALAMUS VACULARITE OF THEIR OFFSPRING

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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ABSTRACT

EFFET OF MATERNAL HIGH FAT DIET ON HYPOTHALAMUS VACULARITE OF THEIR OFFSPRING

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Obesity is a medical condition that carries increased risk for the onset of metabolic disorders and many other chronic diseases. However, etiology of obesity and how obsegenic traits are emerged remain to be elucidated. Investigations about this issue focused on one of the brain regions, hypothalamus, where energy homeostasis, food intake and appetite are regulated. Under the condition of hypothalamic inflammation and/or because of the passage of toxic substances from the circulating blood to the hypothalamus, hypothalamic cells are disrupted and metabolic deregulations might initiate, risk factors for obesity and other cluster of metabolic disorders might increase.

Recent studies have shown that the maternal obesity and/or obesogenic diet consumption during pregnancy may initiate hypothalamic inflammation which

may permanently alter hypothalamic structures and functions in the offspring. Based on evidences that neuro-vascular changes, especially changes in brain blood barrier (BBB) integrity are not only results in passage of toxic substances from circulating bloods but also may initiate or contribute to hypothalamic inflammation and affect microvessel density.

In this study, we investigated changes in BBB integrity of 20 days-old Wistar rat offspring whose mothers were fed with HFD. Our results shows that offspring whose mothers fed with HFD (cafeteria diet) hypothalamic BBB integrity was increased significantly compare to controls. Our results suggest that maternal obesity has impacts on hypothalamic BBB integrity and such changes can inhibit certain crutial signals to reach hypothalamus and initiate cascade of disease conditions as blocking signal homeostasis during development.

Keywords: Hypothalamus, Neuro-vascular Unit, Microvessels, Brain Blood Barrier, Cafetaria Diet, Obesity

ÖZ

AŞIRI YAĞLI DİYET BESLENEN HAMİLE DİŞİ SIÇAN YAVRULARININ HİPOTALAMUS DAMARLARINDA GÖRÜLEN DEĞİŞİKLİLERİN İNCELENMESİ

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Obezite beraberinde birçok metabolik ve kronik hastalıkların oluşmasına neden olan bir hastalıktır. Fakat, obezitenin etiyolojisi ve obezojenik özelliklerin nasıl ortaya çıktığı tam olarak bilinmemektedir. Bu alanda yürütülen çalışmalarda önemli odak noktalarından biri de beyinin eneryi dengesi, beslenme ve iştahı kontrol eden merkezi olan hipotalamustur. Hipotalamusta gerçekleşen inflamasyon ve/veya kanda bulunan toksik maddelerin bu bölgeye geçmesiyle hipotalamusta bulunan nöronların fonksiyonel olarak doğru çalışamazlar. Nöronlarda gerçekleşen bu fonksiyonel bozukluklar metabolizmada değişikliklere neden olur ve devam eden süreçte obezite ve diğer birçok metabolik hastalıkların ortaya çıkma riskini artırabilir.

Son çalışmalar anneye ait obezitenin ve/veya hamilelik sırasında obezojenik besin tüketiminin yavru hipotalamsunda inflamasyonu başlatabileceğini ve yapısında kalıcı değişikler oluşturabileceğini göstermiştir. Bilindiği üzere nörovasküler ünite ve özellikle beyin kan bariyeri (BKB) bütünlüğünde gerçekleşen değişimler kandaki toksik maddelerin geçişine ve bu bölgede inflamasyonun tetiklenmesine neden olabilir ve genel olarak bu değişimler bölgede mikrodamar yoğunluğunu etkileyebilir. Önerilen projedeki hipotezimiz, anneye ait obezite ve/veya hamilelik sırasında tüketilen FYB'ler BKB bütünlüğünde ve mikrodamar yoğunluğunda azalmaya neden olabilir.

Bu çalışmada, annesi FYB ile beslenen 20 günlük Wistar rat yavrularının BKB bütünlüğü incelenmiştir. Ortaya çıkan sonuçlar, annesi FYB (kafeterya diyeti) ile beslenen annelerin yavrularında BKB bütünlüğünde artma olduğunu ortaya çıkarmıştır. Araştırma sonucunda elde ettiğimiz sonuçlar doğrultusunda; anneye ait obezitenin yavru hipotalamus BKB bütünlüğü üzerinde etkileri olabileceğini ve bu gibi değişimlerim gelişim sırasında gerekli sinyallerin hipotalamus' a taşınamaması sebebiyle gelişimsel sinyal hemostazını etkileyerek hastalık kondisyonlarına neden olabileceği ön görülmektedir.

Anahtar Kelimeler: Hipotalamus, Nörovasküler ünite, Mikrodamar, Beyin kan bariyeri, Kafeterya diyeti, Obezite.

To My Parents

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Finally I would like to thank my family for their encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

LIST OF TABLES

TABLES

CHAPTER 1

INTRODUCTION

1.1. Obesity

Obesity is multi-factorial chronic disease. Socio-cultural environment, genetic, physiological, metabolic, behavioral and psychological factors have impact on development of this disease. Recent studies have shown that over 1 billion overweight and 300 million people clinically diagnosed as obese. All over the world, this number increases seriously and threatens next generations. (Turconi and Hellas, 2007)

Generally, with respect to suspected etiology, obesity is classified in three categories: monogenic, syndromic and common obesity. Single gene mutations, especially within the melanocortin pathways, can initiate development of obesity and categorized as monogenic obesity (Orahilly, 2009). On the other hand, more than one genetic mutation, chromosomal abnormalities or other disease conditions might initiate development of obesity. This form of obesity categorized as syndromic obesity (Emanuela *et. al*, 2012).

Common obesity, other than any other obesity subcategories, observed in the general population and constitutes %95 of obese people population. After over nutrition and/or consumption of high fat diets (HFD) balance between energy intake and expenditure disrupted and obesity occurs as metabolic disease condition. The process begins with storage of excess energy in the fat tissue. Then, with increase of fat tissues peripheral inflammation and obesity condition occurs (Gregor and Hotamisligil, 2011). Obesity together with disrupted metabolism, increases possibility of many other diseases: Type2 diabetes,

cardiovascular disease, hepatic steatosis, airway diseases, biliary tract disease, cancer, neurodegeneration and neurodegenerative diseases (Hotamisligil, 2006). With current investigations it is thought that all these can be associated with obesity and obesity decreases not only life quality but also life span (Turconi and Hellas, 2007).

1.1.1. Maternal Obesity and Effects on Offspring

Statistical studies have shown that obesity rate affected all age groups including childbearing ages and this rate increases dramatically with passing years for women (Stotland and King, 2011). Obesity during pregnancy has huge impact on both mother and offspring health during pregnancy. Possibility of hypertension, diabetes, thrombotic complications, pregnancy complications, postpartum hemorrhage and also caesarean section rates increases for mothers with obesity conditions (Prior, *et al.*, 2011).

Currently, although the fatal roles of obesity for both mother and offspring during pregnancy is known, affect of maternal obesity on later life of both mother and offspring is under investigation. Studies on obese mothers have shown that with development of pre-eclampsia and gestational diabetes during pregnancy, after conception, risk of development of diabetes and cardiovascular disease increases dramatically. On the other hand, studies have shown that during pregnancy offspring can be affected developmentally, and later obesogenic features might develop with other metabolic disease features, cardiovascular problems, cognitional and behavioral dysfunctions (Prior, *et al.*, 2011).

1.1.2. Animal Models for Maternal Obesity Studies

In order to investigate obesity and/or affect of maternal obesity on offspring health, studies have developed animal models with trying to generate similar conditions comparable to human obesity. In this field of studies obesity conditions are generally established by using fat and/or carbohydrate ingredient

increased pellets (Buettner *et. al*, 2007). However, some of model animals might resist consuming such pellets and this generates some experimental difficulties. In order to overcome such difficulties, like daily junk food consumption with high fat/energy, a new alternative diet type called cafeteria diet has developed. Generally this type diet contains different type junk foods like biscuits, chips, cookies, cakes, chocolates (**Table 1**). Studies conducted with cafeteria diet have shown that it can cause long-term neuronal changes on brain stems where metabolism and/or appetite-satiety regulated. Therefore, it is thought that for obesity and metabolic disease studies cafeteria diet might be more sufficient than normal high fat and/or carbohydrate diets (Sampey *et. al*, 2011).

Vertebrates have strong energy balance systems and accepted as appropriate animal models. Research conducted on maternal obesity and effect on offspring are generally carried out with these animal models. As initial step obesity phenotype acquired with high fat and/or carbohydrate diets and then, effect of maternal obesity condition on offspring with different developmental stages can be investigated. Maternal studies conducted with vertebrates have uncovered new ideas about etiology of diseases. Currently, it is thought that diseases occurred in older ages might be developed because of maternal reasons during pregnancy and/or during lactation process (Heindel, 2011). Maternal obesity or high fat consumption during pregnancy might be given as example of important risk factors for offspring that can initiate offspring´s obesity, leptin resistance, hypertension, hyperleptinaemia, tachycardia, vascular endothelial dysfunction, hyperglisemia, insulin resistance, and fat accumulation in liver (Prior *et. al*, 2011). Although, many studies have reported affect of maternal obesity on offspring, it is not completely understood how these metabolic features pass through or how metabolic changes are initiated and continues in offspring. Recent investigations, propose that during obesity condition there are changes in the neurological networks and inflammatory signaling observed in the nuclei belongs to hypothalamus, where metabolism controlled (Cheung and Ingraham, 2011).

1.2. Neurobiology behind Food Intake

Sixty one years ago as proposed by Kennedy G.C, 1953, the brain regulates energy homeostasis via controlling circulating signals from blood and adjusting appropriate response. As depicted in **Figure 1** circulating signals; nutrients such as glucose, free fatty acids and hormones from different sources such as leptin from adipose, insulin from pancreas, cholecystokinin (CCK) and glucagon-like peptide 1 (GLP1) from intestine are carried by blood or peripheral nervous system and sensed by brain at specific locations. As such signal transduction mechanisms brain integrates environmental and metabolic information into neuronal signals and activates specific neuronal networks. As a result peripheral information turns into physiological activations to guarantee energy homeostasis (Morton *et. al*, 2014).

After peripheral signals reach the brain, for metabolism homeostasis and food intake almost all brain regions have secondary roles (**Figure 2)** in order to generate and adjust signal to physiological activations appropriately as organizing satiety, appetite, digestion, glucose and fat metabolism and also metabolism rate (Murray *et. al*, 2014 and Koch and Horvath, 2014). However, in order to provide such balance by neuronal networks requires immense physiological responsibilities. Neurons from different nuclei have to protect their plasticity and number. Any factor that affects neuronal numbers, plasticity or neuronal functionality can damage these networks and directly affect metabolic homeostasis for organism (Cai D *et. al*, 2011).

In addition, studies conducted recently have revealed that although almost all brain regions have secondary roles for metabolic homeostasis, hypothalamic nuclei especially Arcuate Nucleus (Arc) have primary roles (Koch, 2014).

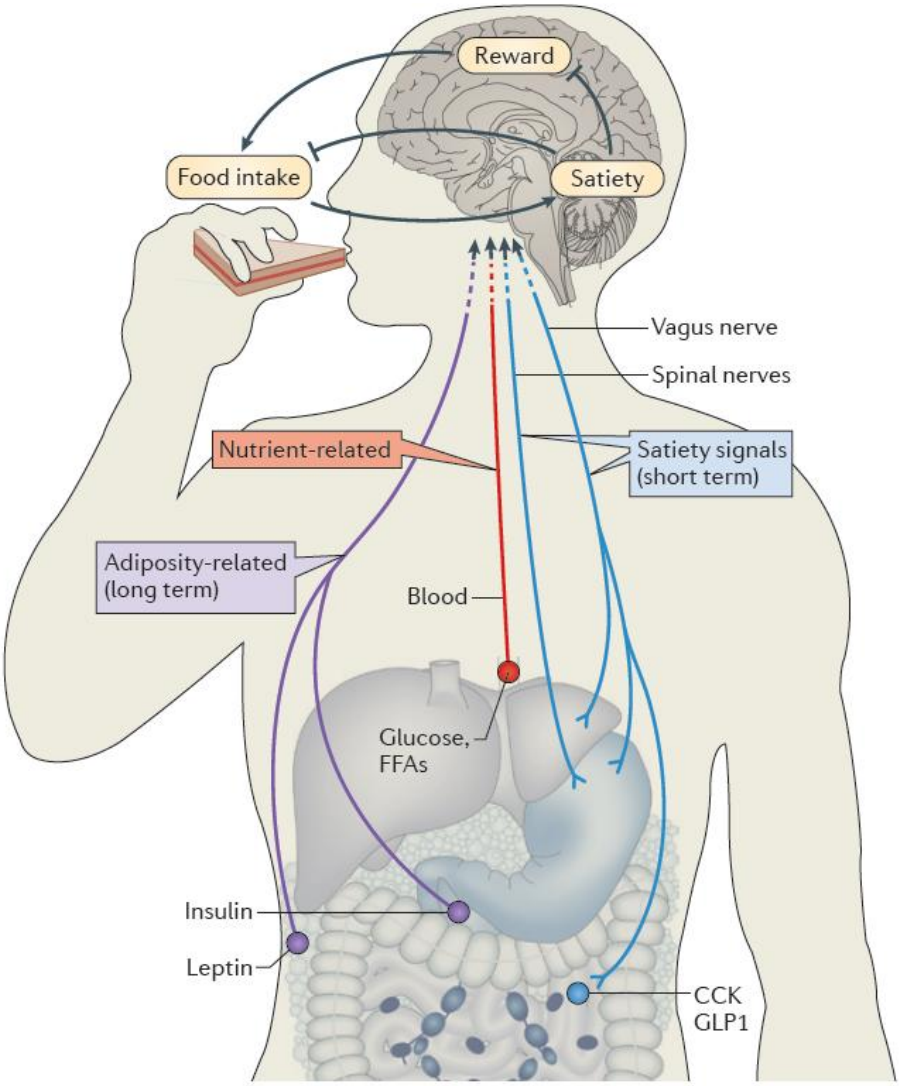

Figure 1. Energy homeostasis regulation via brain: The central nervous system (CNS) integrates input from both peripheral nervous system and blood borne factors: leptin, insulin and nutrition itself. (CCK: cholecystokinin, FFAs: free fatty acids, GLP1: glucagon-like peptide 1) (Morton *et. al*, 2014).

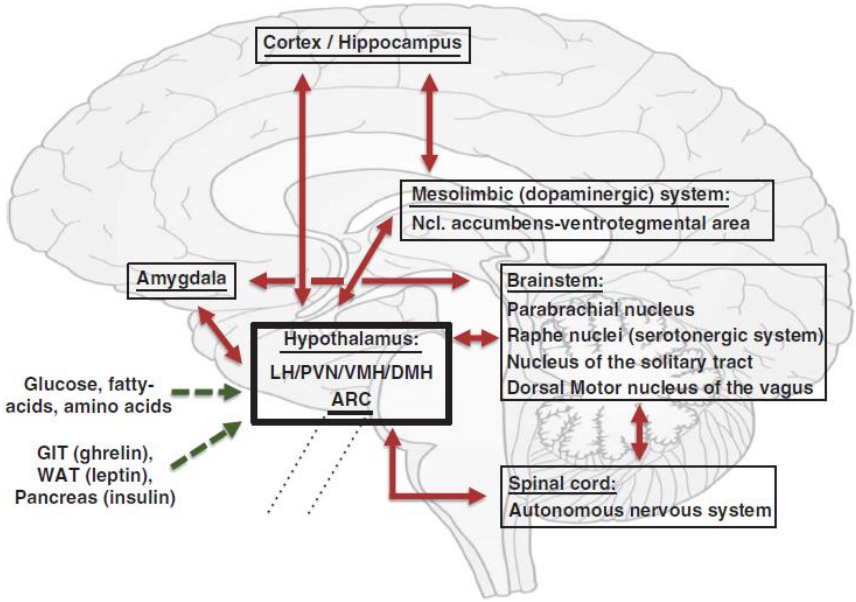

Figure 2. Control of food intake and energy metabolism. Neuronal circuitries: control of food intake and energy metabolism. ARC, arcuate nucleus; DMH, dorsomedial hypothalamus; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus; GIT, gastrointestinal tract; WAT, white adipose tissue. Anorexigenic pro-opiomelanocortin (POMC) neurons and the functional antagonistic, orexigenic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons (GABA, γ aminobutyric acid; MC4R, melanocortin type 4 receptor; ME, median eminence; 3 V, third ventricle (Koch and Horvath, 2014).

1.3. Hypothalamus

Hypothalamus is a part of diencephalon and presents under the thalamus and above the pituitary. Although this area is relatively small, it consists of distinct nuclei (**Figure 3** and **Figure 4**) where different neuron families specialized on different duties about regulation of metabolism. These nuclei consist of 3 zones (periventricular, medial, lateral) or 4 areas anterior to the posterior area (preoptic, supraoptic, tuberal and mamillary). In addition these nuclei, there are

afferent and efferent fibers presents in hypothalamus to get in contact with different brain regions (Braunstain, 2011).

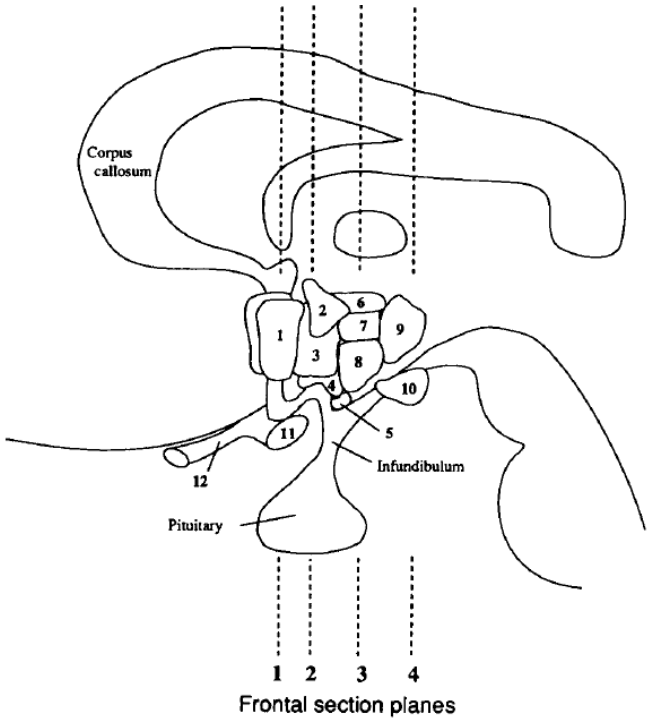

Figure 3. Lateral brain sections demonstrating hypothalamic nuclei. 1, preoptic nucleus; 2, paraventricular nucleus; 3, anterior hypothalamic area; 4, supraoptic nucleus; 5, arcuate nucleus; 6, dorsal hypothalamic area; 7, dorsomedial nucleus; 8, ventromedial nucleus; 9, posterior hypothalamic area; 10, mamillary body; 11, optic chiasm; 12, optic nerve (Melmed *et. al*, 2011).

Functionality of hypothalamus and functional information about each hypothalamic nuclei acquired from different studies, clinical observations, or specifically damaging specific nuclei or triggering these nuclei with different methodologies such as electrical shocks, optogenetical methodologies or designer receptors exclusively activated by designer drugs (DREADD) methodologies (Koch and Horvath, 2014). It is well known that hypothalamus

have roles in water metabolism, body temperature regulations, appetite control, circadian rhythm, energy balance, general metabolism regulation, sleep-wake cycle, control of functionality of visceral organs, behavior, memory, anterior pituitary control and emotional expression (Braunstain, 2011).

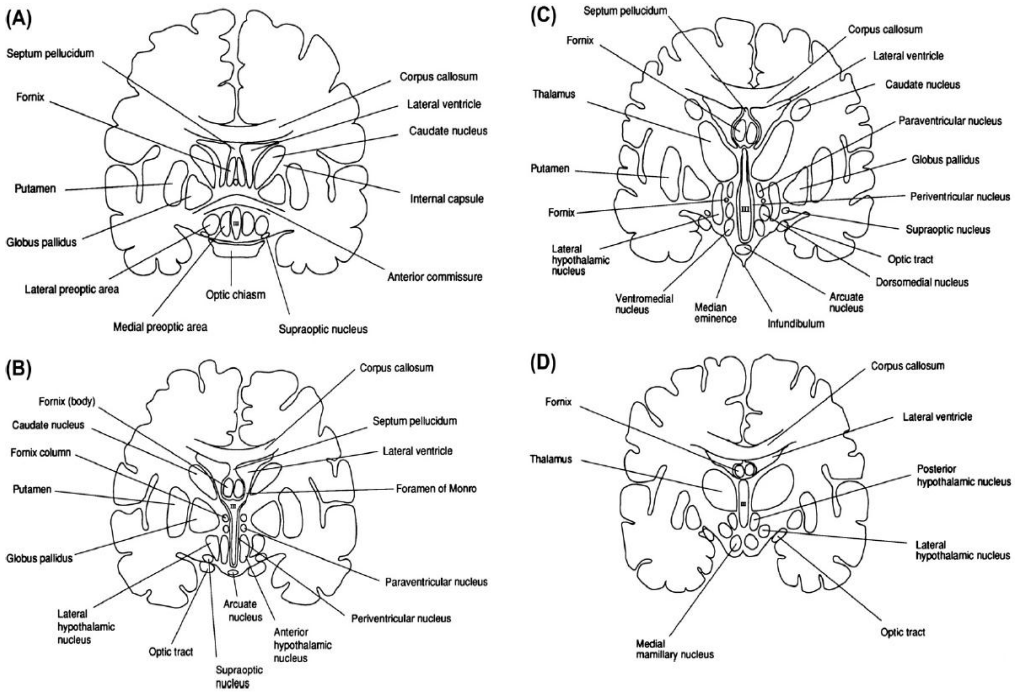

Figure 4. Frontal (coronal) sections of the hypothalamic regions**.** (A) Represents the preopticregion (B) represents the supraoptic.(C) represents the tuberal; (D) represents the mamillary region. (Melmed *et. al*, 2011).

1.3.1. Hypothalamic Control of Food Intake and Energy Metabolism

Energy balance and metabolism control one of the crucial functions of hypothalamus for organisms to survive. After digestion, metabolites and other signals like hormones are carried to hypothalamus via blood circulations and these signals initiate signaling cascades to either initiation of food intake or termination of food intake (Cai *et. al*, 2011). For example, when thyroxin,

glucocorticoids and/or ghrelin reaches to hypothalamus initiates food intake tendency, however, when GLP-1, peptide YY3–36 (PYY3–36) and insulin or leptin reaches to hypothalamus initiates satiety (Stanley *et. al*, 2005 and Coll *et. al*, 2007). Different neuronal groups detect different signals and convert these environmental signals into neuronal and neuro-hormonal signals (**Figure 5**) (Prior, *et al.*, 2011).

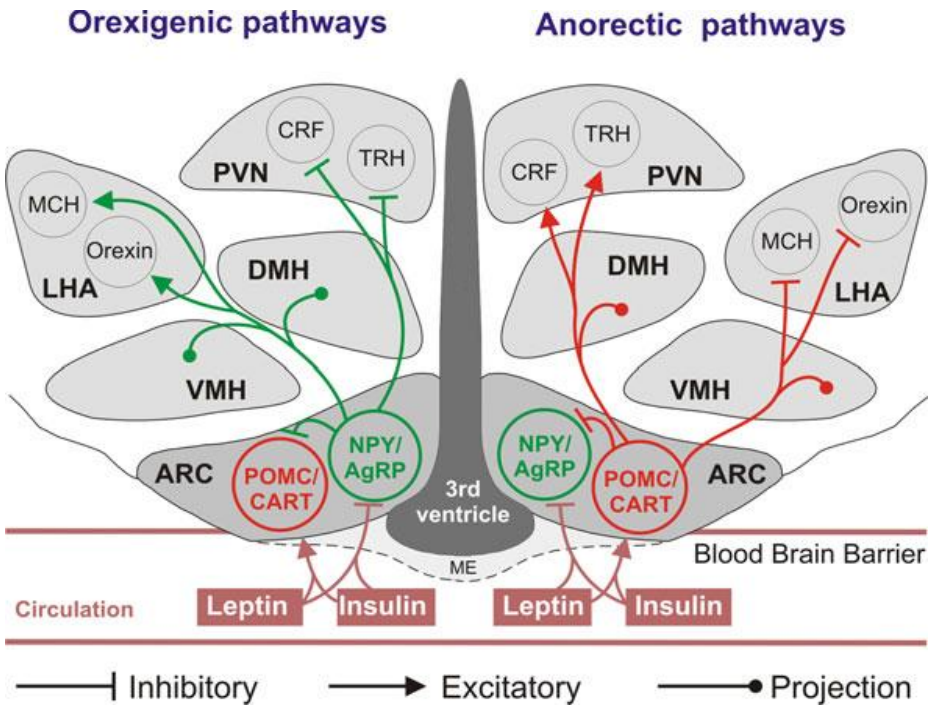

Figure 5. Neural pathways that stimulate appetite or satiety: Orexigenic pathways are green and anorectic pathways are red. Abbreviations: pro-opiomelanocortin (POMC), cocaine and amphetamine transcript (CART), neuropeptide Y (NPY), agouti-related transcript (AgRP), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH) corticotrophin releasing factor (CRF) and thyroid-releasing hormone (TRH), melanin-concentrating hormone (MCH) , median eminence (ME) (Prior *et al*., 2011)

First studies started 74 years ago to identify how hypothalamus controls metabolism and energy balance (Hetherington and Ranson, 1940). With initial lesion studies it is thought that hypothalamic dorsomedial, paraventricular, ventromedial, and Arc were satiety nuclei and lateral hypothalamus was appetite nucleus. However, with recent technological achievements it is realized that these areas have different neuronal populations that have opposite duties (Prior *et. al*, 2011). For example, at Arc there are 4 types of neurons identified recently. Two of them are orexigenic and expresses 2 neurohormones; NPY, AgRP. As opposite manner, other 2 group of neurons are anorexigenic and expresses POMC, CART. When blood borne signals reaches to median eminance (ME) where BBB is not tight, signals are sensed at Arc and from there all neurological signals carried to different hypothalamus nuclei and also different brain areas (**Figure 5**) (Dietrich and Horvath, 2013).

However, these metabolic homeostasis regulated by hypothalamus can be disrupted by affecting neuron groups by inflammation or any other environmental factors that changes functionality of neurons (Cai and Liu, 2011).

1.3.2. Affects of High Fat Diet on Hypothalamus

Studies have shown chronic over nutrition or high fat diet initiates premise inflammation and associated stress signals at the hypothalamus and later develops hypothalamic inflammation (**Figure 6** and **Figure 7**). In addition to these studies, Thaler *et al.*, 2012 have shown that hypothalamic inflammatory gene expressions initiates before peripheral inflammation after high fat diet within 3 day. Because first inflammation occurs at hypothalamus, it is thought that obesity and cascade of other metabolic diseases initiates with hypothalamic neuronal dysfunctions together with deregulation of metabolism (**Figure 7**) (Cai *et. al*, 2011; Zhang *et. al*, 2008; Possey *et. al*, 2009; Kleinders *et. al*, 2009; Purkayastha *et. al*, 2011; Ozcan *et. al*, 2009; Sabio *et. al*, 2010; Belgard *et. al*, 2010; Kievit *et. al*, 2006; Milanski *et. al*, 2009; Meng *et. al*, 2011).

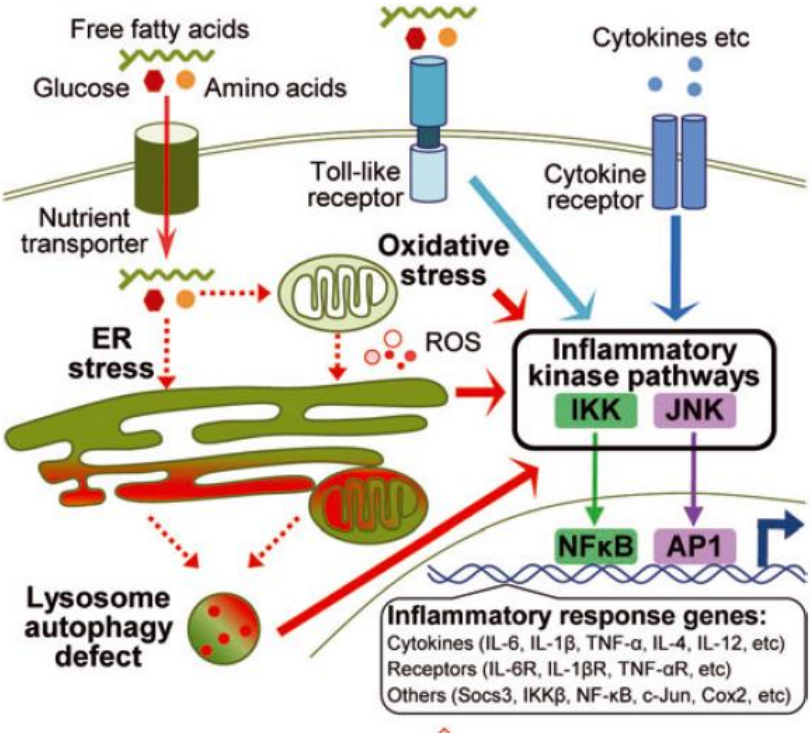

Figure 6. Metabolic inflammation in the hypothalamus and signaling cascades: Under the chronic over-nutrition and high fat diet; oxidative stress, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and late-stage lysosome autophagy defect are induced. Stress conditions activate proinflammatory regulators;IkB kinase (IKK) and c-Jun N-terminal kinase (JNK) and nuclear transcription factors NF-kB or AP1 to initiate gene expression of inflammatory response molecules. (Cai and Liu, 2011).

1.3.3. Effect of Maternal Obesity on Offspring Hypothalamus

Studies conducted on obesity and HFD revealed hypothalamus have crucial roles on development of metabolic diseases. However, these studies have been carried out generally with adult animals. With recent studies, it is realized that not only HFD consumption or over-nutrition can affect hypothalamus negatively

but also offspring hypothalamus can be affected because of maternal obesity and HFD consumption during pregnancy. These studies have shown that maternal obesity and HFD consumption during pregnancy changed offspring hypothalamic physiology including some of gene expressions (Chang *et. al*, 2008). These early changes might result in long-term hypothalamic physiological changes and cause metabolic dysregulations for offspring. Because of these reasons investigations about physiological and molecular mechanisms behind hypothalamic dysfunction are crucial (Cheung and Ingraham, 2011).

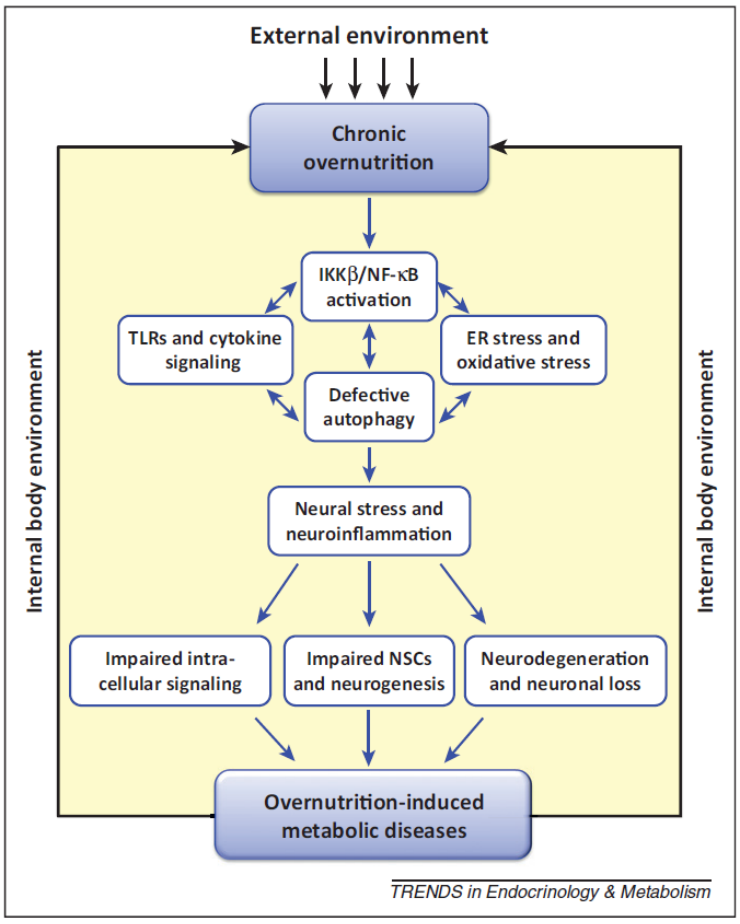

Figure 7. Effect of overnutrition on hypothalamic nervous system. Abbreviation: TLRs, toll-like receptors. NSCs, neural stem cells (Cai, 2013).

1.4. Communication between Blood Vessel and Neurons

Blood vessels and nerve fibers form networks throughout the organism in order to achieve their complexity to ensure that all tissues receive an adequate blood supply and a proper connection to the central nervous system. As this way, informational processing and nutritional support can be acquired.

With recent studies mechanism involved in wiring neural and vascular networks seem to share some deep similarities (**Figure 8**) (Carmeliet and Tessier, 2005). Angiogenesis (blood vessel formation) factors have roles in neurogenesis and neural network formation during development process and it is crucial force for shaping nervous system and protecting it from diseases (Greenberg and Jin, 2005).

Similarities and high communication between blood vessels network and nervous system are also observed within brain. This communication not only shapes brain structure but also protects neurons and provides appropriate environment for neurons to function properly (Zlokovic, 2008 and Stanimirovic and Friedman, 2012).

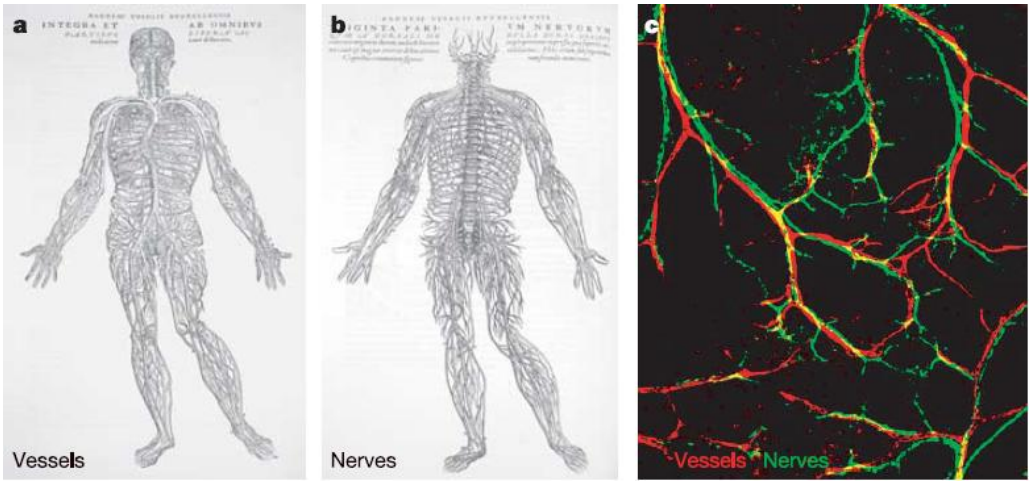

Figure 8. Nerves and Blood Vessels (Carmeliet and Tessier, 2005).

1.4.1. Neurovascular Unit

Throughout the centuries because of being conductive unit, and having key roles neurons are accepted as main target for brain disorders and brain pathologies. However, it was realized that in order to have healthy brain functionalities cell types other than neurons within brain; astrocytes, oligodendrocytes, microglias, pericytes, and also microvascular endothelial cell have also crucial roles. Neurons and these cell types have high communications and their communication is very important for healthy neuron functionality. As a functional unit, neurons, pericytes, astrocytes and microvessel endothelial cells forms neurovascular unit (**Figure 9**) (Lok *et. al*, 2007). Together with neurovascular pathology hypoxia at tissue, inflammation activations, leaky BBB, and also communicational dysfunction between astrocytes, oligodendrocytes, microglias, pericytes, and also microvascular endothelial cell are observed. This situation results in neurovascular disintegration, neuronal dysfunctions and also brain regional damages (Stanimirovic and Friedman, 2012).

In addition, neurovascular unit members; astrocytes, oligodendrocytes, microglias, pericytes, and also microvascular endothelial cells as forming negative and positive feedback communications generate brain blood barrier (BBB) (Zlokovic, 2008). BBB is composed of tightly sealed vascular endothelial cells (**Figure 10**) by tight, adherence and gap junctions. BBB is specialized neurovascular unit structures and together with astrocytes, microglias and pericytes control passage of factors from blood and prevents toxic substances and molecules to enter brain. Through this mechanism neuronal environment is protected (Zlokovic, 2008).

Investigations about neurological diseases have recently focused on whether roots of neurological diseases derive from brain vascular pathologies; for example, Alzheimer's disease, dementia, epilepsy, migraine, multiple sclerosis. Acquired or inherited vascular diseases can generate those neurological disease

conditions. Also, it was demonstrated that when communication within neurovascular unit is disrupted, dysfunction of neurogenesis neuronal degeneration and functional problems can be observed (Stanimirovic and Friedman, 2012).

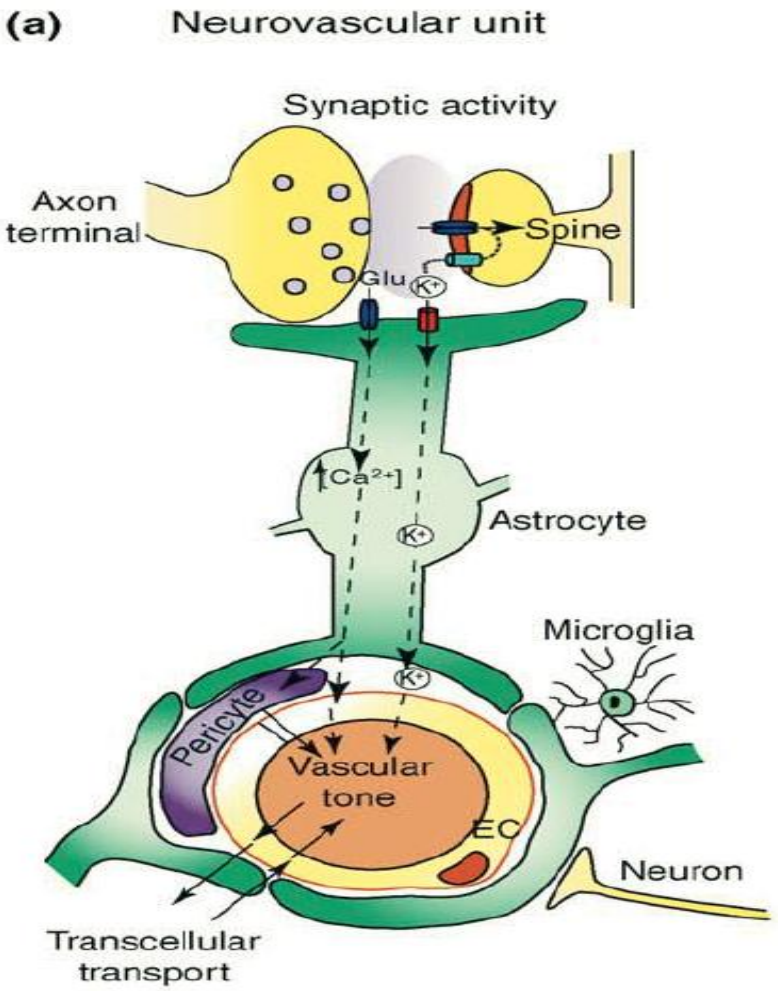

Figure 9. Neurvascular Units (Quaegebeur *et. al* , 2010)

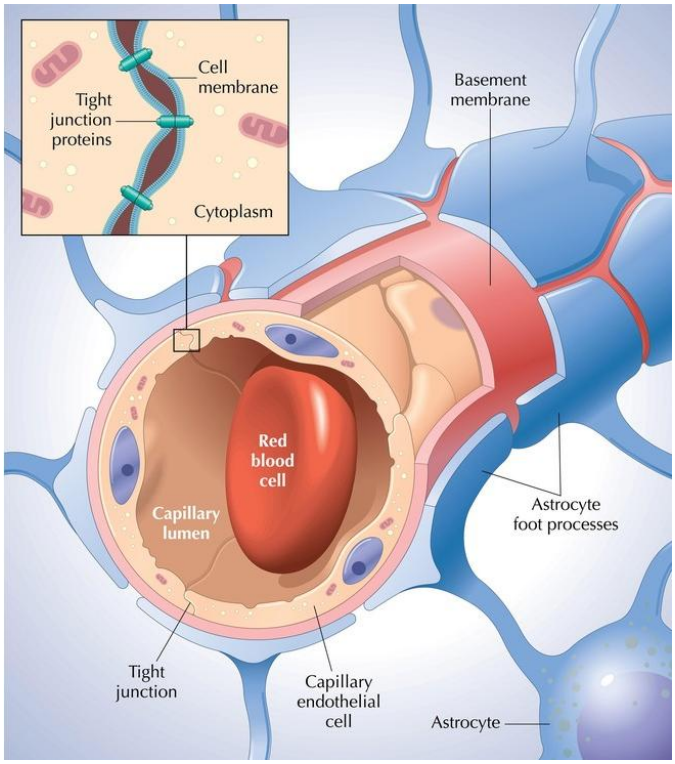

Figure 10. Brain Blood Barrier (Perkins J., 2014)

1.4.2. Affect of HFD on Brain Microvessels

Affect of HFD on peripheral vascularity is known relatively, however its affect on brain microvessels is not understood completely (Freeman and Granholm, 2012). While investigating synaptic physiology with electron microscope it was noticed that microvessels are also affected by HFD (**Figure 11)** (Horvath *et. al*, 2010). Also, several groups have shown that changes could occur in the BBB integrity after HFD and cholesterol diet at the hippocampus region (**Figure 12**) (Freeman and Granholm, 2012).

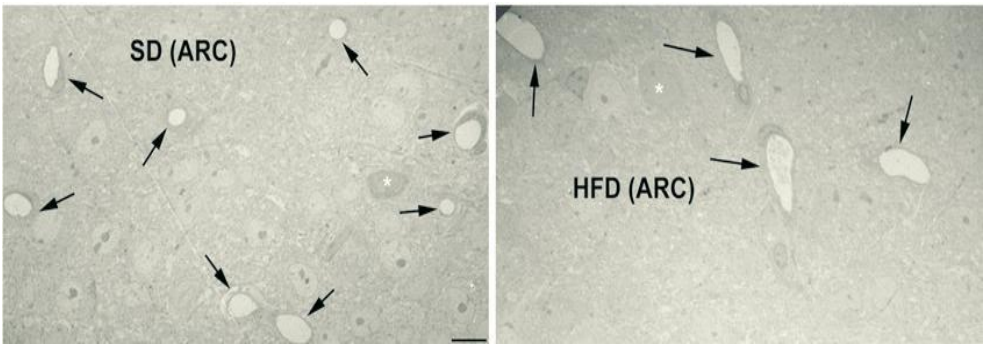

Figure 11 Affect of HFD on Arc nucleus. SD, standard diet. HFD, high fat diet. Arrows shows vessels (Horvath *et. al*, 2010).

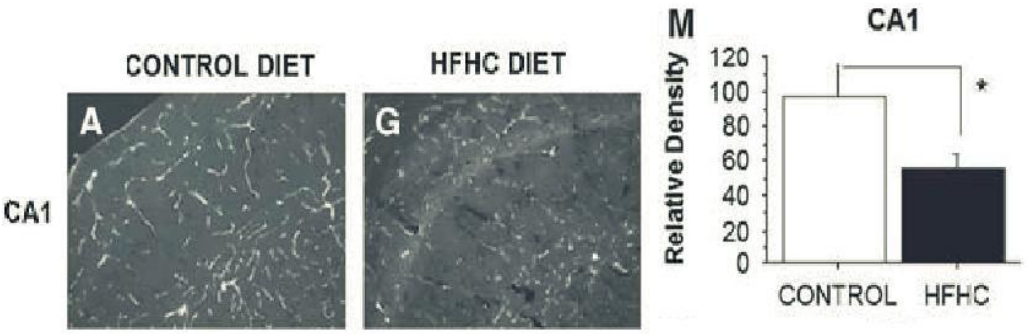

Figure 12 BBB integrity change at hippocampal CA1 region: BBB integrity investigated with immuno-flourescent staining using anti-SMI-71 (Freeman and Granholm, 2012).

1.5. Aim of the Study

Hypothalamus is one of the most important brain region for regulation of metabolism and metabolic homeostasis. Changes in hypothalamic structures or signaling within this region can directly trigger metabolic deregulations and might initiates metabolic disorders. Therefore, most studies conducted about etiology of obesity and other metabolic disorders focused on this region (Cai, 2011). Because of being conductive unit and having key roles in signal transduction, neurons are accepted as main target for neurological roots of metabolic diseases in the hypothalamus (Cai, 2013).

Metabolic disease studies focused on neurological roots revealed three main mechanisms about hypothalamic neurons; defective autophagy, endoplasmic reticulum (ER) stress and inflammatory signaling cascade (**Figure 7)** (Cai, 2013). However, although all these mechanisms have great impact on hypothalamic neuronal dysfunctions; brain vascularity, especially NVU, have immense amount of roles for neurons to function properly (Stanimirovic and Friedman, 2012; Zlokovic, 2005).

Currently, the role of brain vascular changes in the metabolic disease conditions and affects of maternal obesity on offspring brain vascularity are remained to be studied. Therefore, it was aimed to study whether maternal obesity, hence, maternal metabolic disease conditions may affect offspring hypothalamic vascularity.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animal Studies

Experiments were initiated with 5 weeks-old female Wistar rats, total of 20 animals were used which were divided as n=10 rat/group. In order to acquire obese phenotype, the rats were fed either cafeteria diet as HFD,called the HFD group or normal/chow (standard diet, SD), called the control group for 8 months. Ingredients and composition of the diet were specified at Table 1. Until weaning (3 weeks after childbearing) all groups (mothers) were continued on their diet as cafeteria diet or control. At the end of the weaning all groups were returned back to normal chow diet. The offsprings and adult male rats which were used for mating were not fed with cafeteria diet, they were on SD.

After 3 weeks of gestation period, generally from each mother approximately 8 to 12 offspring were born. From each group; both control and HFD n=10 offspring were sacrificed at 0th, 10th, 20th, 30th and 90th days. Number of sacrificed offspring and the time table were shown in **Figure 13.**

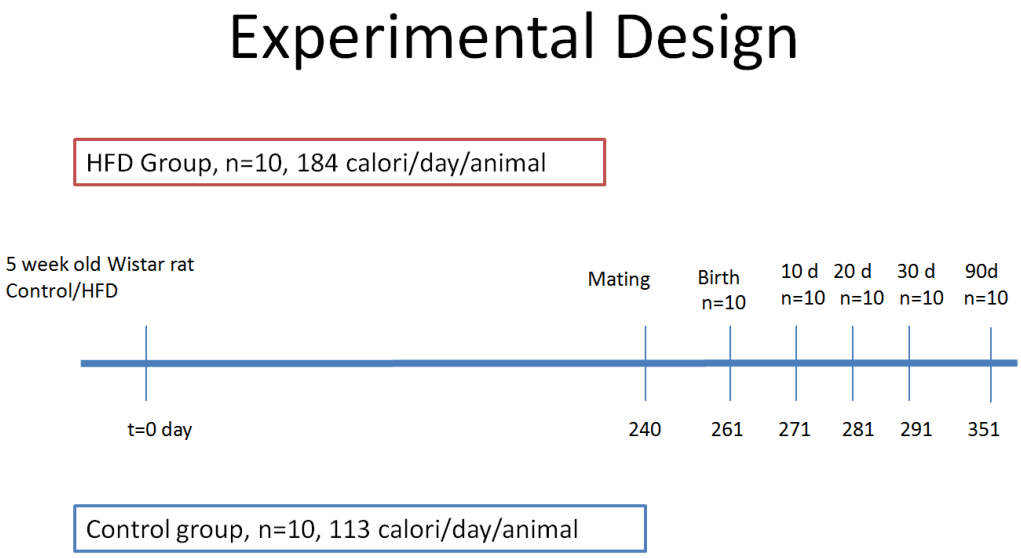

Figure 13. Experimental design and average calorie intake for the HFD and control groups (n=10 rat/group). HFD feeding process had been continued for 8 months. From each mother group n=10 offspring were sacrificed at different ages (0 th, 10 th ,20 th, 30 th and 90 th days).

2.2. Dissection of Brain Tissues

Offspring were exposed for 10 sec. to CO_2 and then decapitation was performed with guillotine. Isolated brains were directly immersed into liquid nitrogen and stored at -80°C.

In order to localize hypothalamus and Arc from the whole brain, 3D simulation (**Figure 14)** and also the brain atlas (**Figure 15**) were used. Between -4,08mm/- 1,80mm from bregma was sectioned according to the brain atlas (Paxinos, *et al*., 2007).

The hypothalamic sections were acquired with the cryostat instrument at different thicknesses; 5, 10, 20, 30, 40 and 50 µm (Leica- SM2010R, UNAM, Bilkent Univ.) and placed onto glass slides

Table 1: Cafeteria Diet Composition

Energy and Food Ingredients (100 g)	**Total (kcal)**	**Total fat (g)**	**Total Carbohydrates (g)**	**Protein (g)**	**Sugar (g)**
Normal Yem: SC 7001 (Harlen)	382	4	54	25	0
Çay Keyfi (Eti)	462	20.4	67.8	5.8	28.5
Hoşbeş (Eti)	493	24.5	63.9	7.6	28.4
Hanımeller (Ülker)	427	18.1	62.1	3.9	25.0
Doritos (Frito-Lay)	491	24.5	60.5	7.2	2.3
Lays Klasik (Frito-Lay)	529	33	51	7.0	0
Pringles	515	33	51	4.0	3.2
Crunch (Nestle)	486	26	69	6	51
Burçak (Eti)	450	17.7	71	7.2	20.4
Lays Wavy (Frito-Lay)	536	36	54	7	0
Nesquik mısır gevreği (Nestle)	372	4.1	76.1	7.6	30.7
Kombo (Eti)	481	21.7	68.5	6.2	29.6
Çizi (Ülker)	473	21.4	64.3	5.8	0

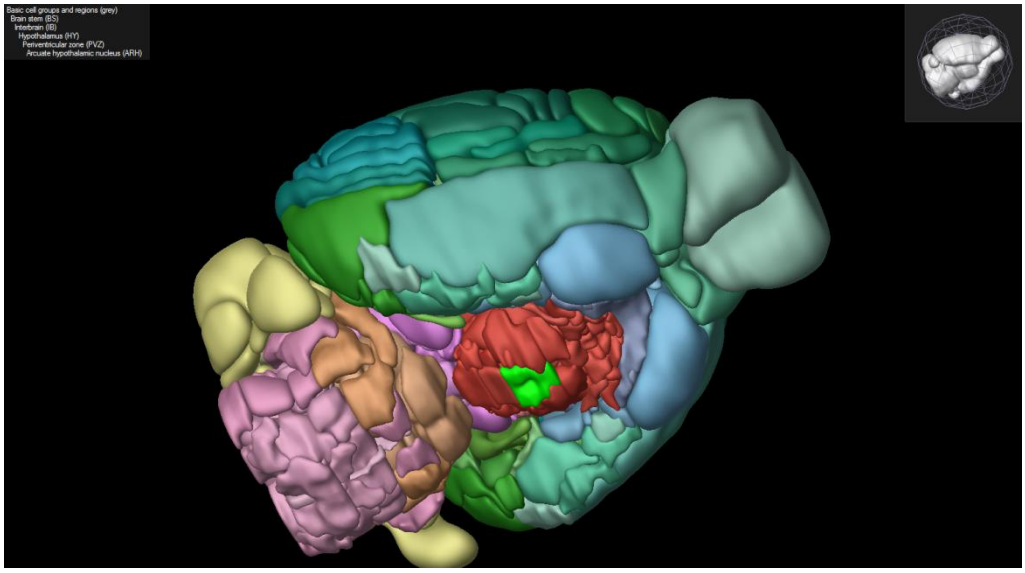

Figure 14. 3D brain simulation developed by Allen Institute for Brain Research, USA. Red area: hypothalamus and Green area inside the red area: Arc nucleus.

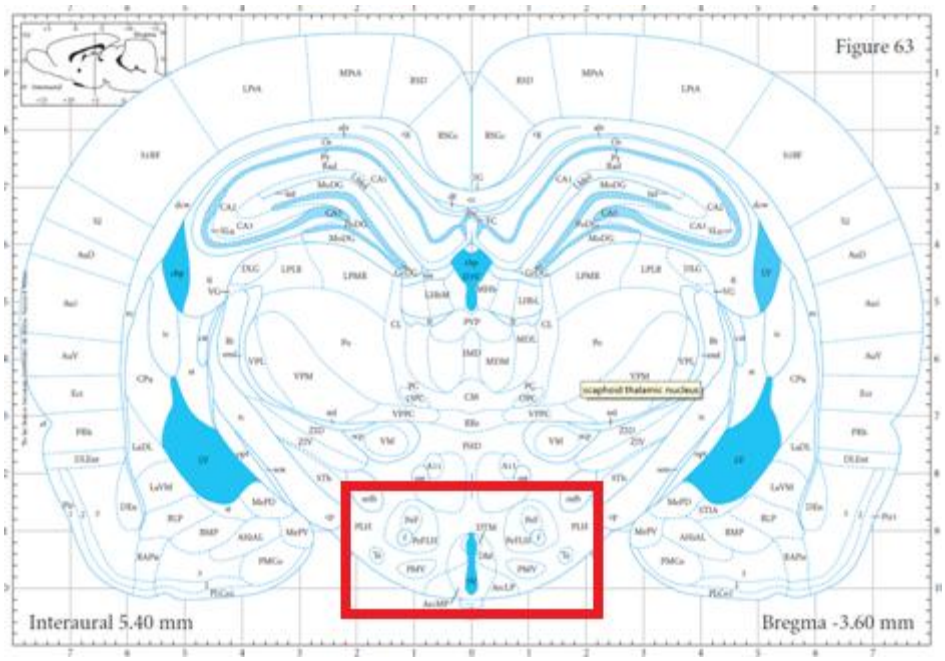

Figure 15. The section from rat brain atlas. From bregma: -4,08mm / - 1,80mm which indicates the hypothalamic area (the red box) (Paxinos and Watson, 2007).

2.3. Antibodies

To investigate hypothalamic microvessel BBB integrity anti-SMI-71 (Convence, USA) primary antibody and DyLight®550 secondary (Abcam, USA) antibody were used for immunoflourescence (IF). SMI-71 is specific for an endothelial protein found in areas with blood-brain or blood-nerve barriers. The antibody does not react with endothelia of periventricular organs or with fenestrated endothelia in peripheral tissues. Reactivity with the antibody develops in newborn rats along with maturation of the blood brain barrier (Abcam).

To investigate microvessel density or neovascularization anti-CD31 (Abcam, USA) primary antibody and DyLight®488 (Abcam, USA), secondary antibody were used.

2.4. Immunoflourescence (IF) for Frozen Brain Tissues

As an initial step, tissues were fixed at acetone (or Ethanol) 5 min at 4°C and then 5 min at room temperature (RT). Next, they were placed into PBS 3X, 5 min each. After the washing step, tissues on the glass lams were enclosed using hydrophobic Pap Pen. Then, 100 ul a protein block was loaded onto thoseand incubated 1 h at RT. Later, slides were placed into PBS for 1-2 sec. After the washing step, 100 ul primary antibody was loaded onto tissues and incubated overnight at 4°C. Next day, slides were washed 5X, 3 min each with TBS which followed by secondary antibody (100 ul) incubation for 30 min at RT. Finally, slides were washed with PBS 3X, 5 min each and were closed with flouro-shield mounting medium (Abcam, USA).

All solutions were detailed at Appendix.

Imagings of the tissues (both 2D and 3D) were acquired from laser scanning confocal microscope (Zeiss LSM 510, (Thornwood, NY, USA), UNAM, Bilkent

Univ. The quantification of the IF images were obtained from 3D images acquired from the confocal microscope (40 micron, objective 20X) and ImageJ programme was used to calculate the stained area

2.5. Glucose Tolerance Test

The rats were removed from their cages and placed in fresh cages with water but without food for overnight (approximately 12-18 hr). After this period, first fasting glucose levels (time 0) were measured abd the tail blood was dropped onto a hand-held Accu-Chek Compact glucometer (Roche Diagnostics, USA). Then, 30% Dextrose solution was administered (ip, 2 gr/kg body weight) to rats. Next, glucose levels were measured with compact glucometer at 5, 10, 15, 30, 60, and 120 min after injection.

2.6. Offspring Pancreatic Islet Isolation and Analysis of Glucose Response

20 and 90 day old offspring pancreatic islets were isolated by pancreatic duct injection of collagenase type V solution. Viability of islets are analyzed with propidium Iodide (PI) and florocein diasetat (FDA).Islets were incubated in 3.3mmol/l glucose for low glucose concentration. For high glucose; the islets were incubated in 16.7mmol/l. The solutions (low glucose and high glucose) were collected for insulin ELISA assay.

2.7. Statistical Analysis

Images were quantified with the Fiji program and statistical analysis was performed with the GraphPad Prism 6 program. For all data sets, the arithmetic average, standard deviation (STD) and standard error of the mean (SEM) were computed by the GraphPad Prism 6 program. Error bars depicted SEM. To examine the significance of the data sets the Student's t-test was used. Data were considered as significant with $^{*}p<0.05$ and as highly significant with $^{**}p<0.01$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Before Gestation Body Weight

After 8 months of cafeteria diet, there was statistically significant weight difference between the control and HFD group mothers before gestation (**Figure 16).** As a result, the average weight of HFD group was 570g (**Figure 17**) and the average weight of the control group was 310g (**Figure 18)** (****p< 0.0001 from the control group). Therefore, the HFD group was considered obese.

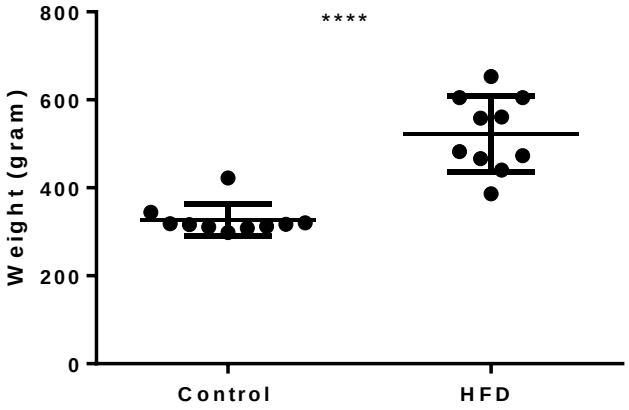

Figure 16: Weight (g) difference of mothers before gestation (n=10 rat/group). The average weight of the control group was 310g and the HFD group was 570g (****p< 0.0001 from the control group).

Figure 17: Before gestation, female rats in the HFD group with cafeteria diet.

Figure 18: Before gestation, a female rat in the control group.

After 8 months of feeding the animals, they were sacrificed and observed that increased intra-abdominal adiposity surrounding the peripheral organs (**Figure 19**) vs. to the control group (**Figure 20**). Liver color of HFD rats was changed to a pale color compared to the controls, Figure 19 and 20, respectively.

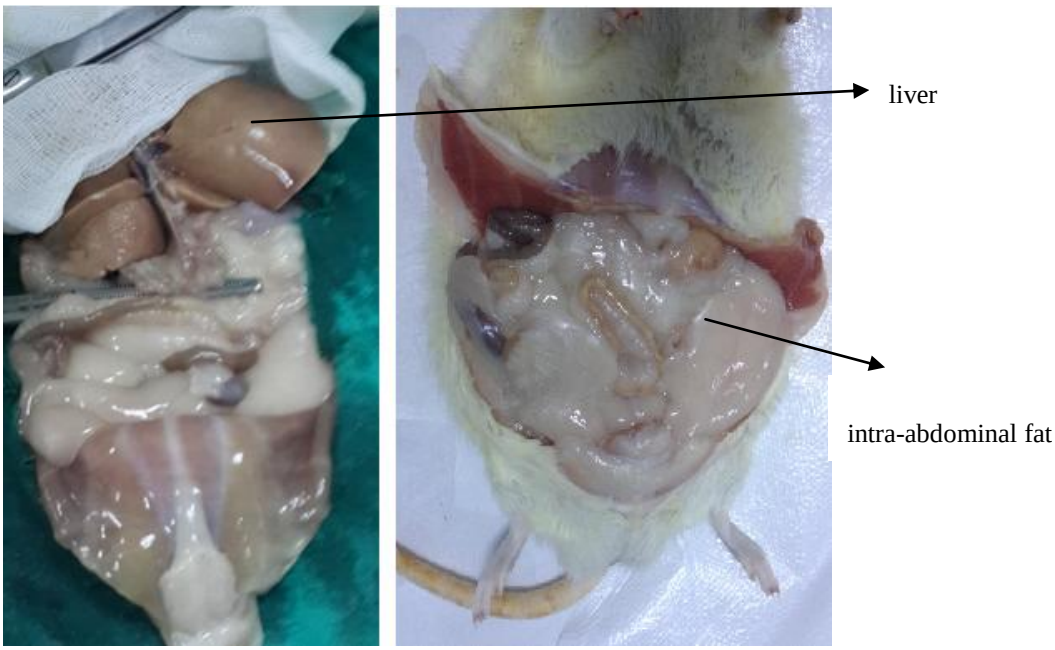

Figure 19. After 8 months of cafeteria diet for the HFD group, the intra-abdominal fat was increased that surrounded peripheral organs.

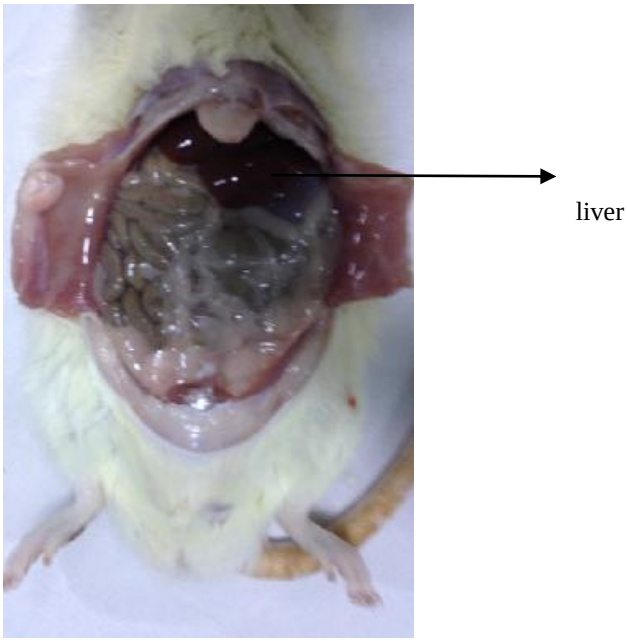

Figure 20. After 8 months of normal/chow diet for the control group, the intra-abdominal fat was not increased compare to the HFD group and the peripheral organs were not surrounded with fat.

3.2. Development of Type2 Diabetes Before Gestation for Mothers

The HFD group developed Type 2 diabetes after 8 months (Figure 21). Based on the glucose tolerance test blood glucose levels of the HFD group did not decrease below 200 (mg/dl) after 2h which might be indicative of the existence of insulin resistance; hence, development of Type2 diabetes compare to the control group (****p< 0.0001 from the control, n=10 rat/group) (Figure 21)

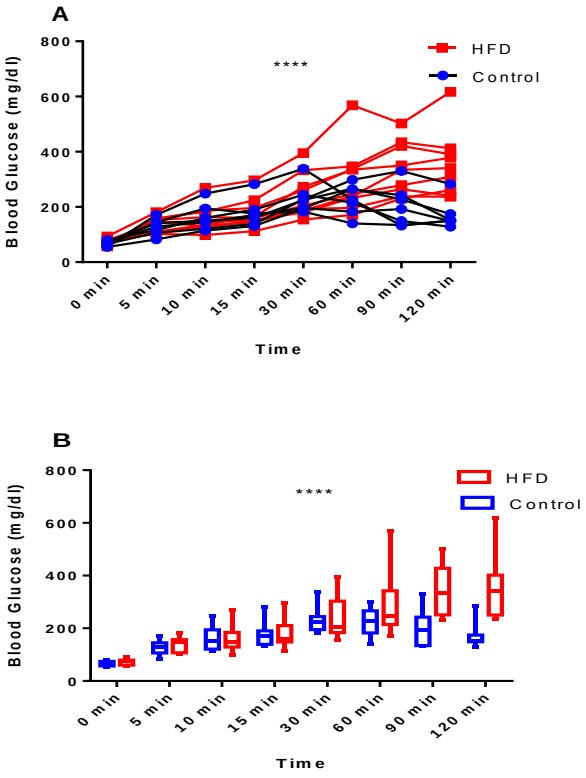

Figure 21. Glucose tolerance test for both the control and HFD group mothers. Each individual animal indicated in A. Mean with maximum and minimum values depicted in B. In the HFD group, glucose was not cleared from blood after 120 min. (****p< 0.0001 from the control (Two-way ANOVA)).

3.3. Hypothalamic BBB Integrity of 20 Day-Old Offspring

In this experiment 20 days-old offspring´s hypothalamic brain regions were analyzed(n=5). Both 2D (Figure 23, the HFD group and Figure 24, the control group) and 3D (Figure 25, the HFD group and Figure 26, the control group) IF images showed that HDF and control groups offspring´s hypothlami were different from each other on the BBB integrity. To quantify the stained and average values acquired using the Image J programme. The quatification data showed that the BBB integrity was increased significantly in the HFD group vs. to controls (**Figure 22**) (n=5 rat/group ***p*<0.01 from the control group).

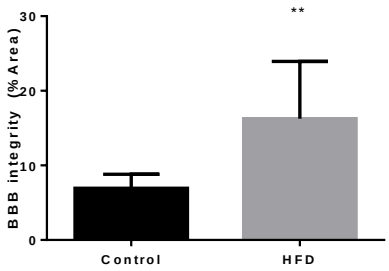

Figure 22. Quantification of the BBB integrity of the hypothalamus in the HFD group vs. control group offspring (20 day old offspring). 3D images acquired from the confocal microscope (40 micron, objective 20X). As using ImageJ programme the stained area was acquired (n=5 rat/group***p*<0.01 from the control group. Mean ± SEM of column A 6,937 ± 0,7690, Mean ± SEM of column B 16,26 ± 3,135)

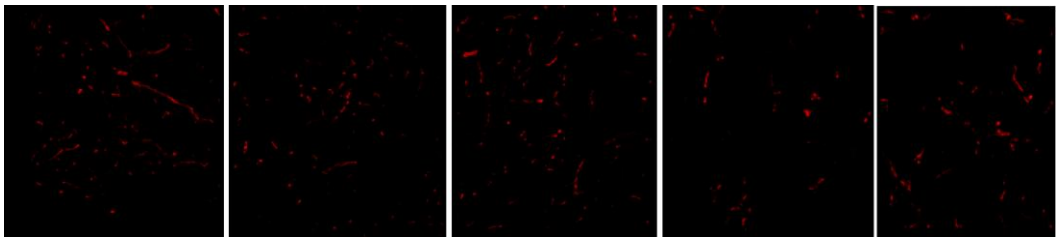

Figure 23. Immunoflourescence images of the control group 20 days-old offspring´s hypothalamus for the BBB integrity. The tissues were probed with anti-SMI-71 and images acquired by confocal microscopy (40 micron, 20X objective). (n=5, each panel represents n=1).

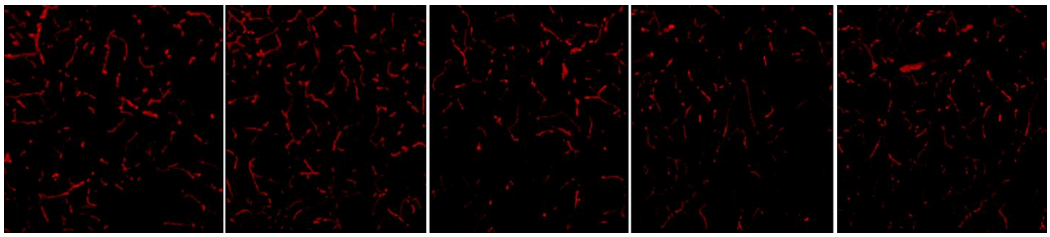

Figure 24. Immunoflourescence images of the HFD group 20 days-old offspring's hypothalamus for the BBB integrity.The tissues were probed with anti-SMI-71 and images acquired by confocal microscopy (40 micron, 20X objective) (n=5, each panel represents n=1).

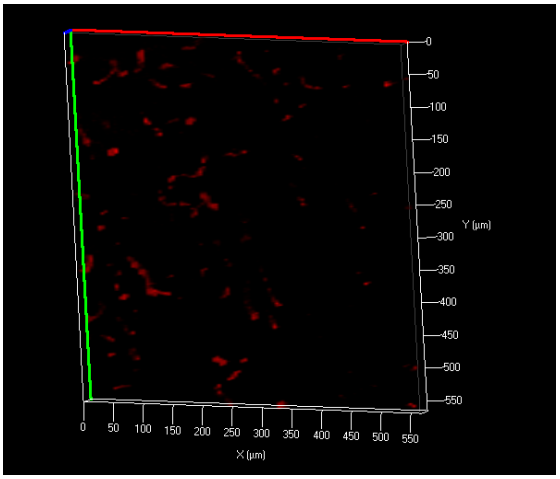

Figure 25. 3D image of the control group 20 days old offspring´s hypothalamus for the BBB integrity. Images were obtained by confocal microscopy and (40 micron, objective 20X) and tissues were probed with anti-SMI-71.

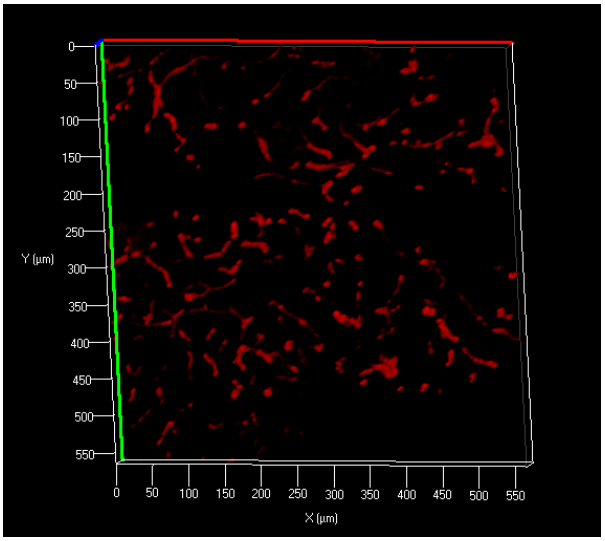

Figure 26. 3D image of the HFD group 20 days old offspring´s hypothalamus for the BBB integrity. Tissues were probed with anti-SMI-71. and mages were obtained by confocal microscopy (40 micron, objective 20X).

3.4. Effects of Maternal Diet On 20 Day-Old Offspring Pancreatic Islets

From 20 day old HF diet and control group offspring, islets were isolated and glucose-induced insulin secretions are compared. Trend in insulin response between HF diet and SC diet groups were significantly different. HFD group offspring islets highly responded low and high glucose with respect to control group offspring islets.

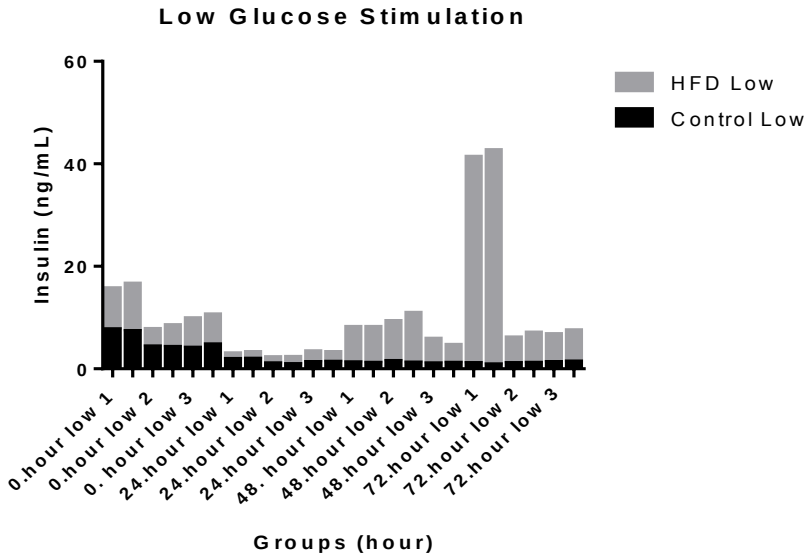

Figure 27. Primary islet cell culture isolated from 20 days old HFD and control groups offspring. Isolated primary islet cell response to low glucose (3.3mmol/l) measured with Insulin ELISA assay after 0, 24, 48, and 72 hour. Each assay repeated 3 times.

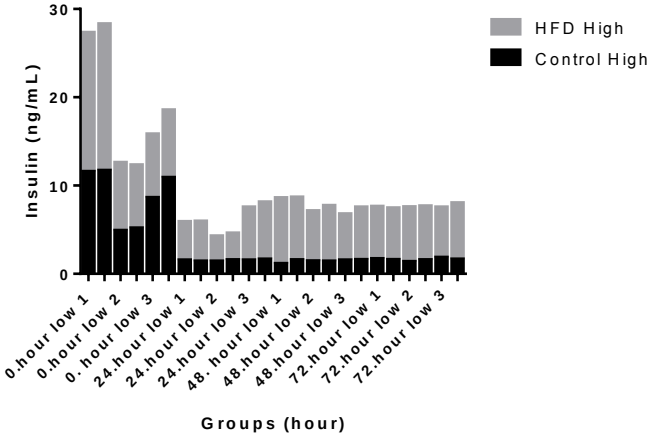

Figure 28. Primary islet cell culture isolated from 20 days old HFD and control groups offspring. Isolated islet cells response to high glucose (16.7mmol/l) measured with Insulin ELISA assay after 0, 24, 48, and 72 hour. Each assay repeated 3 times.

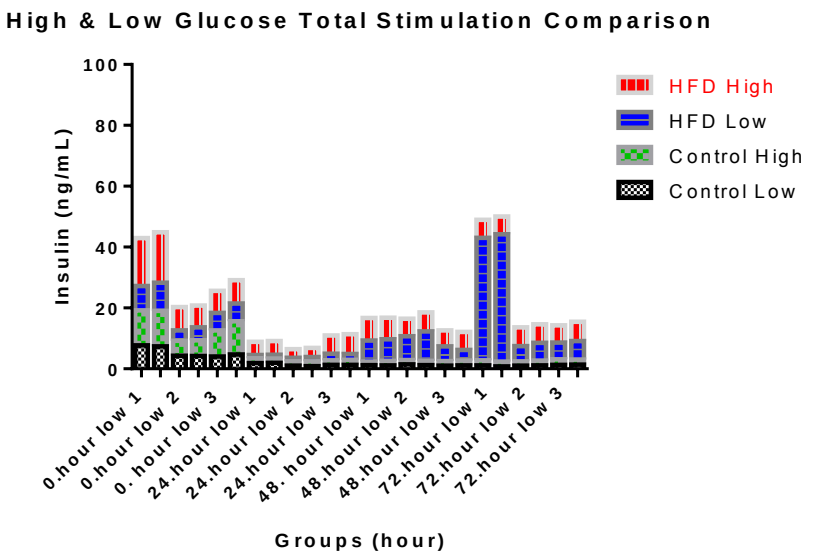

Figure 29. Primary islet cell culture isolated from 20 days old HFD and control groups offspring. Isolated islet cells response to high glucose(16.7mmol/l) and low glucose(3.3mmol/l) measured with Insulin ELISA assay after 0, 24, 48, and 72 hour. Each assay repeated 3 times.

3.5. Hypothalamic BBB Integrity of 90 Day-Old Offspring

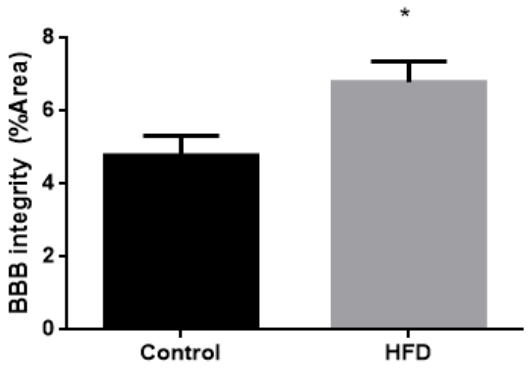

Figure 30. Quantification of the BBB integrity of the hypothalamus in the HFD group vs. control group offspring (90 day old offspring). 3D images acquired from the confocal microscope (40 micron, objective 20X). As using ImageJ programme the stained area was acquired (n=3 rat/group**p<0.01 from the control group. Mean ± SEM of column A 4,786 ± 0,3090, Mean ± SEM of column B 6,799 ± 0,3277)

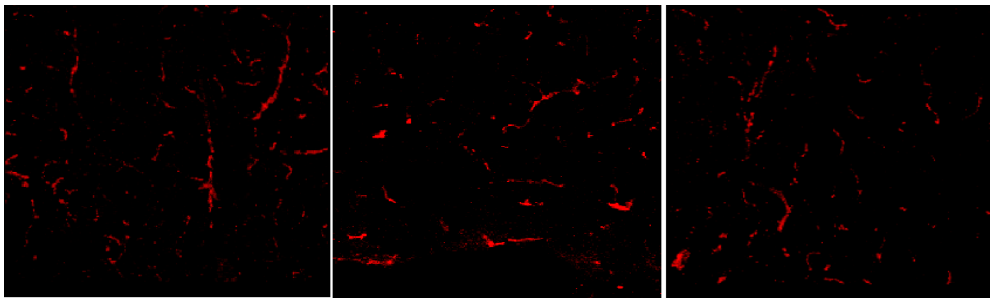

Figure 31. Immunoflourescence images of the control group 90 days-old offspring´s hypothalamus for the BBB integrity. The tissues were probed with anti-SMI-71 and images acquired by confocal microscopy (40 micron, 20X objective). (n=3, each panel represents n=1).

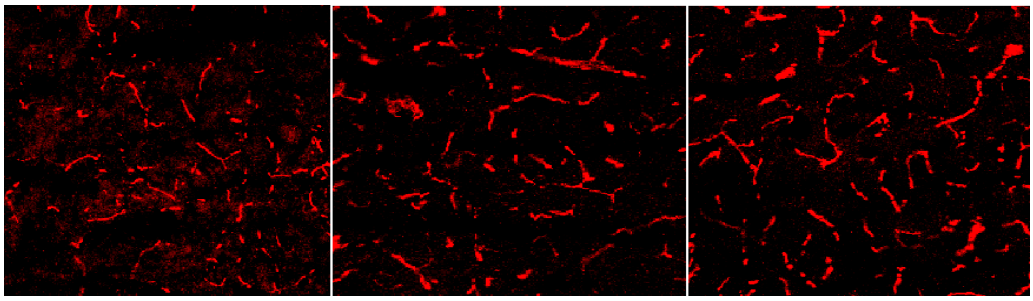

Figure 32. Immunoflourescence images of the HFD group 90 days-old offspring's hypothalamus for the BBB integrity.The tissues were probed with anti-SMI-71 and images acquired by confocal microscopy (40 micron, 20X objective) (n=3, each panel represents n=1).

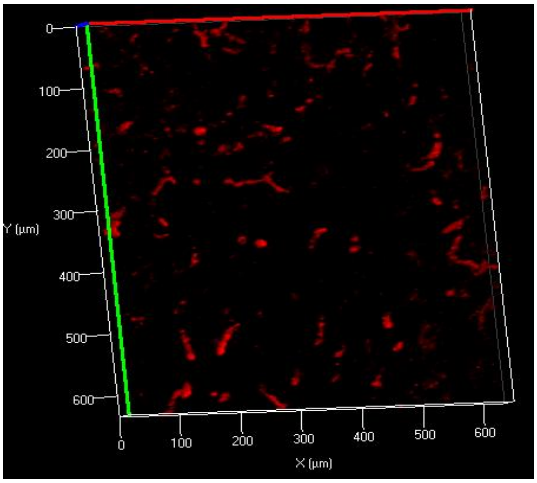

Figure 33. 3D image of the control group 90 days old offspring´s hypothalamus for the BBB integrity. Images were obtained by confocal microscopy and (40 micron, objective 20X) and tissues were probed with anti-SMI-71.

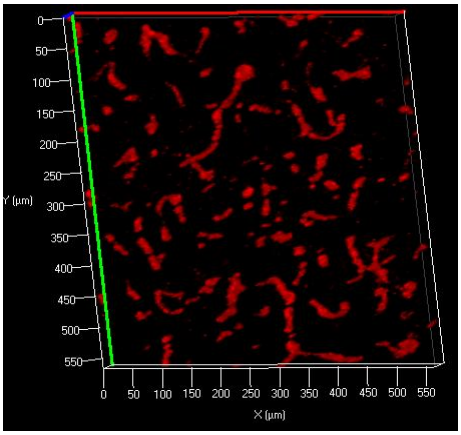

Figure 34. 3D image of the HFD group 90 days old offspring´s hypothalamus for the BBB integrity. Tissues were probed with anti-SMI-71. and mages were obtained by confocal microscopy (40 micron, objective 20X).

3.6. Effects of Maternal Diet On 90 Day-Old Offspring Pancreatic Islets

From 90 days old HF diet and control groups offspring, islets were isolated and glucose-induced insulin secretions were compared. Trend in insulin response between HF diet and SC diet groups were significantly different. The HFD group offspring islets highly responded low and high glucose vs. to control group offspring islets.

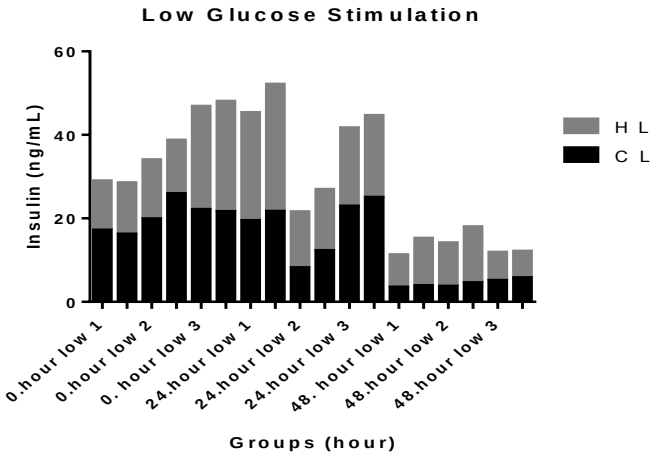

Figure 35. Primary islet cell culture isolated from 90 days old HFD and control groups offspring. Isolated primary islet cell response to low glucose(3.3mmol/l) measured with Insulin ELISA assay after 0, 24, 48, and 72 hour. Each assay repeated 3 times.

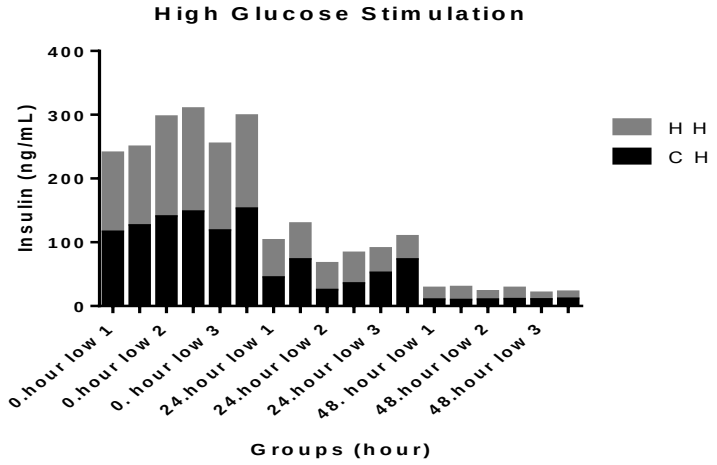

Figure 35. Primary islet cell culture isolated from 90 days old HFD and control groups offspring. Isolated primary islet cell response to low glucose(3.3mmol/l) measured with Insulin ELISA assay after 0, 24, 48, and 72 hour. Each assay repeated 3 times.

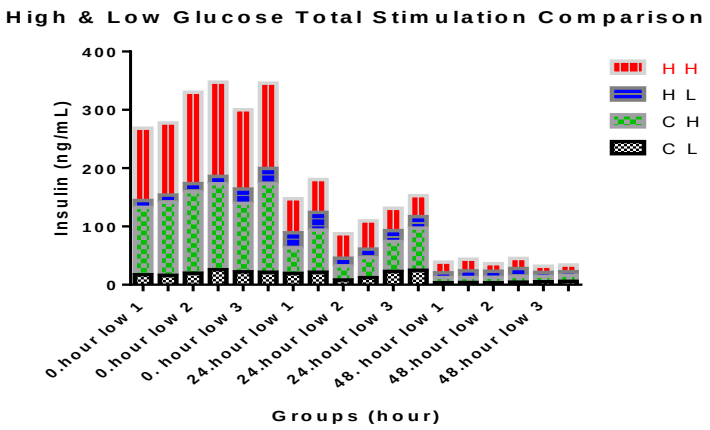

Figure 36. Primary islet cell culture isolated from 90 days old HFD and control groups offspring. Isolated primary islet cell response to low glucose (3.3mmol/l) measured with Insulin ELISA assay after 0, 24, 48, and 72 hour. Each assay repeated 3 times.

3.4. Analysis of Hypothalamic Microvessel Intensity

Twenty days-old offspring´s hypothalami from both HFD and control groups were stained with anti-CD31 to analyze microvessels intensity or neovascularization. **Figure 27** for the control group and **Figure 28** for the HFD group showed that the antibody did not properly work and images were with heavy background.

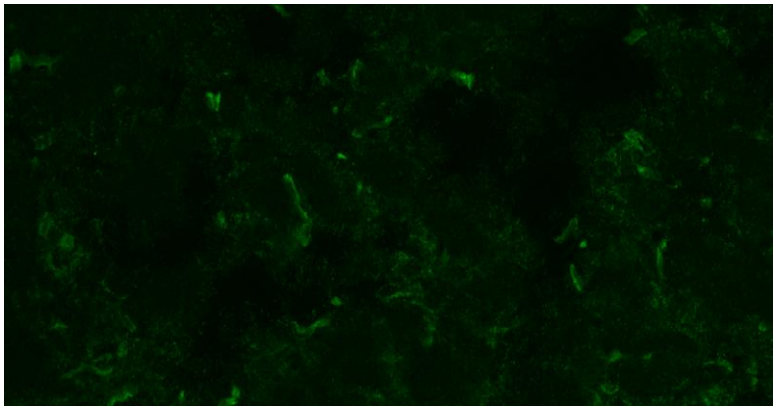

Figure 38 The control group 20 days old offspring hypothalamus microvessel intensity. The tissues were probed with anti-CD31 and images were obtained by confocal microscopy (5 micron, objective 40X). .

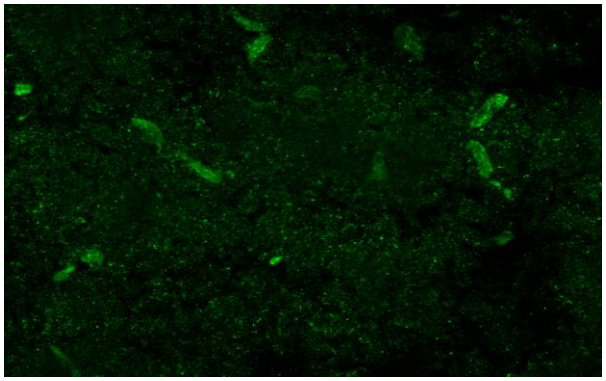

Figure 39 The HFD group 20 day old offspring´s hypothalamus microvessel intensityTissues were probed with anti-CD31 and images were obtained by confocal microscopy (5 micron, objective 40X).

CHAPTER 4

CONCLUSION

Obesity is a medical condition and threatens world population seriously. Other than monogenic and syndromic obesity, common obesity highly observed in any human population (%95) in recent years. However, although the incidence rate is high, basic biological roots of this subcategory is remained to be solved. In order to elucidate etiology of common obesity, recent studies focused on the role of physiological factors, especially the brain hypothalamus region and disease mechanisms within this region (Cai, 2011).

Studies conducted about hypothalamus revealed that hypothalamus have roles in water metabolism, body temperature regulations, appetite control, circadian rhythm, energy balance, general metabolism regulation, sleep-wake cycle, control of functionality of visceral organs, behavior, memory, anterior pituitary control and emotional expression. Among them, metabolism homeostasis is one of the crucial roles of hypothalamus and any reason affect functionality within this region might initiate metabolic deregulations and result in metabolic diseases (Cai, 2013).

Statistical investigations have shown that incidence of obesity increased in all age group including child bearing ages for women. Recent studies have shown that maternal obesity and/or or high fat consumption during pregnancy can initiate metabolic deregulations in the offspring and affect hypothalamus physiology seriously (Vogt *et al*., 2014). However, physiological and molecular mechanisms behind these changes and how these obesogenic features pass from mother to offspring are not known completely (Prior *et al*., 2011).

With current studies, it was realized that the neuro-vascular system (NVU) might have crucial roles for healthy nervous system and provide protection of this the network from any diseases. NVU provides appropriate niche for neuron stem cells and extracellular environment for neurons to function properly. However, if any acquired and inherited change occurs at the BBB and/or communication between NVU members, damage occurs within the local area and these types of damages mimics and/or contributes to generation of neurological diseases (Stanimirovic and Friedman, 2012). Currently, studies about affects of obesity and metabolic diseases on the brain vascularity is under investigation and affect of maternal obesity on offspring´s BBB and NVU are not studied yet.

In this study, we compared hypothalamus BBB integrity for 20 and 90 days-old Wistar rat offspring whose mothers were fed with either HFD and normal/chow. Our results showed that offspring whose mothers fed with HFD (cafeteria diet) BBB integrity was increased significantly compared to controls.

Connected to these studies, same animals, 20 days old offspring and including 90 days-old offspring's pancreatic islets were isolated and glucose-induced insulin secretions were performed at Dışkapı Hospital, Ankara. Trend in insulin response between HFD and control groups were significantly different. The HFD group offsprings´ islets highly responded to low and high glucose with respect to control group offspring islets (Appendix A and B, **Figure 27, 28, 29** and **Figure 35, 36, 37**).

In fact, aberrant BBB permeability is a hallmark pathology of many central nervous system diseases (Stanimirovic and Friedman, 2012) as oppose to what was detected in this study for both 20 and 90 days-old offspring hypothalamic BBB integrity. Although abnormal insulin secretion may generate Type2 diabetes or obesity, increase in the BBB integrity might be evaluated as natural tendency towards protection of brain from further damages during offspring development.

On the other hand, increased BBB integrity might also prevent certain crutial signals, metabolites, hormones to reach hypothalamus and initiate cascade of disease conditions as blocking certain signal transduction and signal homeostasis during development (Vogt *et al*., 2014).

Our results suggest that maternal obesity has got great impacts on offspring metabolism especially on pancreatic insulin secretion and also BBB integrity. Increase in the BBB integrity might be have roles as initial brain protection mechanism, however increased physical barrier function may also affect other signal to reach hypothalamus and might initiate metabolic deregulations and developmental brain abnormalities.

CHAPTER 5

RECOMMENDATIONS

Our preliminary studies have shown that maternal obesity and/or maternal metabolic disease condition may affect offspring hypothalamic BBB integrity and pancreatic response to glucose and increase circulating insulin and other hormone levels. In addition, mother milk content also changes with diet (Vogt *et al*., 2014) such as some metabolite levels and/or hormone levels might increase. Offspring brain have to be protected from these hormones and metabolites because after certain level these molecules can have toxic affect on brain (Zlokovic, 2008).

As future studies, after HFD and acquired obesity, same experiments ought to be conducted with male adult Wistar rats to investigate changes in the BBB integrity for the adult onset obesity rather that affect of maternal obesity. Also, molecular mechanisms can be elucidated behind the increased BBB integrity of offspring, to do so hypothalamic microvessels can be isolated and with molecular biology techniques such as microarray analyses can be used to study genes involved in these processes.

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APPENDIX A

IF SOLUTIONS

1X PBS

0.24g KH_2PO_4
1.44g Na_2HPO_4
800 ml ddH_2O
8g NaCL
0.2g KCl

1X PBS-T(0.1%)

500 ul Tween-20
500 ml 1X PBS

%4 Paraformaldehid

50 ml 1X PBS
2 gr paraformaldehid powder

Blok Solition

20 ul Tween-20
4 ml 1X PBS
4 ml %2 BSA

Mounting Solution

Flouroshield Mounting Medium (abcam, ab104135).