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Effect Of Folate Deficiency On Expression Of Proteins On The Mtor Signaling Pathway In The Brain Of C57/bl6 Mice

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EFFECT OF FOLATE DEFICIENCY ON EXPRESSION OF PROTEINS ON THE mTOR SIGNALING PATHWAY IN THE BRAIN OF C57/BL6 MICE

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_____

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DEDICATION

I would like to dedicate this to thesis to my family and friends, especially my sister and all of the other people in my life that believe in my dreams and aspirations.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude towards my advisor Dr. Ahmad R. Heydari for providing his time, effort, resources and support in the successful completion of this thesis. In addition, I would like to thank Dr. Archana Unnikrishnan for playing the role of a mentor and providing her valuable guidance, time and support throughout the completion of this thesis. I would also like to thank my committee members Dr. Heydari, Dr. Diane Cabelof and Mary Width for reviewing my work. A sincere thanks goes out to John Sorge, thank you for being a wonderful lab partner, you really made the long days of research easier. You truly are the definition of a best friend- thank you for all of your guidance and support throughout my research and completion of my thesis. One other person in the lab that supported me through all aspects of my research is Tom Prychitko, thank you very much for all of your direction and support. I would also like to acknowledge Safa Beydoun for providing her valuable time to help me conduct my experiments successfully. Finally I would like to acknowledge the directed study students in my lab that I had a pleasure of teaching and working with: Michael Fitzgerald, Tina Kakish and Aaron Sabal.

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CHAPTER 1: INTRODUCTION

Nutrigenomics is the scientific study of the specific way in which specific genes interact with bioactive food components and affect the genome without altering DNA structure [1]. Nutrigenomics allows nutritionists and microbiologists to elucidate the mechanisms by which nutrients have an impact on the genome [1]. It is important to investigate how several cellular processes in the human body can be modified by foods that interact with gene expression. Nutrition affects brain structure, function and development throughout the life cycle and can have profound implication on cognition, mental health and degenerative diseases [2]. Neurodegenerative diseases affect more than 30 million people throughout the world causing mental disability and death. There are essential and nonessential nutrients that are known to have an impact on metabolism, hormonal balance, energy balance, cell cycle signaling and regulation, apoptosis and angiogenesis [3]. Dietary folate has become an interesting food component to study in the rising field of nutrigenomics. Folate deficiency has been studied extensively due to its association with chronic diseases, such as Alzheimer's disease, cancer and cardiovascular disease [4].

A. Folate

Folate is an essential water soluble vitamin that plays a role in several mechanisms and pathways in relevance to etiologies of birth defects and chronic diseases. Folate or folic acid is found to be available in most plant based foods, however it is often found to be under-consumed in the diets of individuals that eat low amounts of fruits and vegetables [5]. Folate is found in our diets as bioactive forms of fundamental B vitamins [6]. Folate is naturally available in foods as "pterolypolyglutamates", and it is used in vitamin supplements and fortified food products in the form of "monoglutamate folic acid" [6]. Folic acid is the most stable and fully oxidized form of

folate and has a greater bioavailability, therefore it is the form used in fortified foods [7]. Folic acid acts as a coenzyme in the metabolism of amino acids and nucleic acids and plays an integral role in many cellular pathways, DNA and RNA synthesis, cellular replication, intracellular signaling and regulation of cell division [6, 7]. All of these processes are implicated in fetal and placental growth and development; therefore it is crucial to maintain the levels of this vitamin throughout the early stages of development and growth [6, 8]. For this reason, in 1998 the United States and Canada mandated folic acid fortification in staple food items such as most grains and cereal products [8].

B. Neural Tube Defects (NTDs)

Poor folate status in women of child-bearing age and pregnant women is known to cause NTDs, miscarriages, and premature births [8]. Neural tube defects involve the incomplete development of the embryonic structure that becomes the brain and/ or spinal cord; there are two common types of NTDs: spina bifida and anencephaly [9]. This defect occurs when the brain and spinal cord are left susceptible due to the due to the failure of the neural tubes closing [10]. Anencephaly is the most severe and spina bifida can cause a range of morbidities; which includes the paralysis of the lower limbs and urinary and fecal incontinence [11].

The exact mechanism by which folate reduces the risk for NTDs still remains as a part of active research [11].The British Medical Research Council conducted a randomized control trial to determine the effectiveness of folic acid supplementation in the prevention of recurrence of NTDs; it was observed that by taking 4000 micrograms of folic acid the risk of NTD recurrence was reduced by 70% [11]. By 1992 the U. S Public Health Service recommended that women of child bearing age should consume 400 µg of folic acid daily through supplementation and diet to prevent NTDs [11]. However encouraging women to consume 400 µg of folic acid through

supplementation has limitations due to the fact that almost more than 50% of pregnancies in the United States are unplanned [11]. Therefore in 1998 a mandatory folic acid fortification was implemented to provide 140µg per 100g to all enriched cereal grain products and had been estimated to provide 100-200µg of folic acid to women of childbearing age [11]. A significant decrease in the prevalence of NTDs has been observed across the United States and many other countries since the mandatory implementation in 1998 of folic acid fortification [11].

C. Potential Adverse Effects of Folate Fortification

There are always concerns regarding adverse effect consequences to any type of public health intervention as it becomes critical to monitor any and all fortification programs to address any type of emerging concerns. With folic acid fortification, the main concerns that have been monitored are: 1.) Masking of B12 Deficiency Anemia, 2.) Cancer and Epigenetic Changes and 3.) Unmetabolized Folic Acid.

1.) *Masking of B12 Deficiency Anemia*

Early case reports suggested that the consumption of folic acid in amounts <5000µg daily could possibly mask Vitamin B12 deficiency by preventing the development of anemia. If masked and not diagnosed at an early stage, this masking could allow for the progression of Vitamin B12 deficiency related neuropathies. However several studies done prior to and after fortification, suggest that there is very little evidence of neuropathies [11].

2.) *Cancer and Epigenetic Changes*

Studies indicate that a diet high in the consumption of folate through fruits and vegetables can reduce the risk of certain types of cancers. As a result of this assumption studies are being conducted to elucidate the mechanism by which folate impacts epigenetic patterns. Folate plays a key role in two important epigenetic modifications-- DNA synthesis and methylation. Since

folate is involved as a one carbon methyl group in DNA methylation, studies are focusing on how it can positively or negatively affect any disease, especially tumorgenesis [11]. Some studies hypothesize that an early and adequate exposure to folic acid may prevent the development of tumor cells through the provision of enough methyl groups during DNA methylation. However other studies show that a higher intake of folic acid may promote the growth of pre-existing tumors [11]. Nonetheless, further studies must be conducted to determine the way in which folic acid has an impact on epigenetic patterns through a mechanistic approach.

3.) *Unmetaoblized Folic Acid*

An excess intake of folic acid can result from an increased consumption of fortified supplements and foods (such as cereal grain products). If the body exceeds the capacity to metabolize folic acid, the folic acid will be found circulating in the blood [12]. Thus far it has been hypothesized that unmetablized folic acid is related to cognitive decline and impairment among seniors [12, 13].

D. Folate One-Carbon Metabolism

As stated earlier, folic acid plays an integral role in many cellular pathways, including DNA and RNA synthesis, cellular replication, intracellular signaling and regulation of cell division [6, 7]. Folate is the central focus of the one-carbon metabolism pathway and plays in integral in the regulation of the pathway. The one- carbon metabolism pathway is displayed in the figure below:

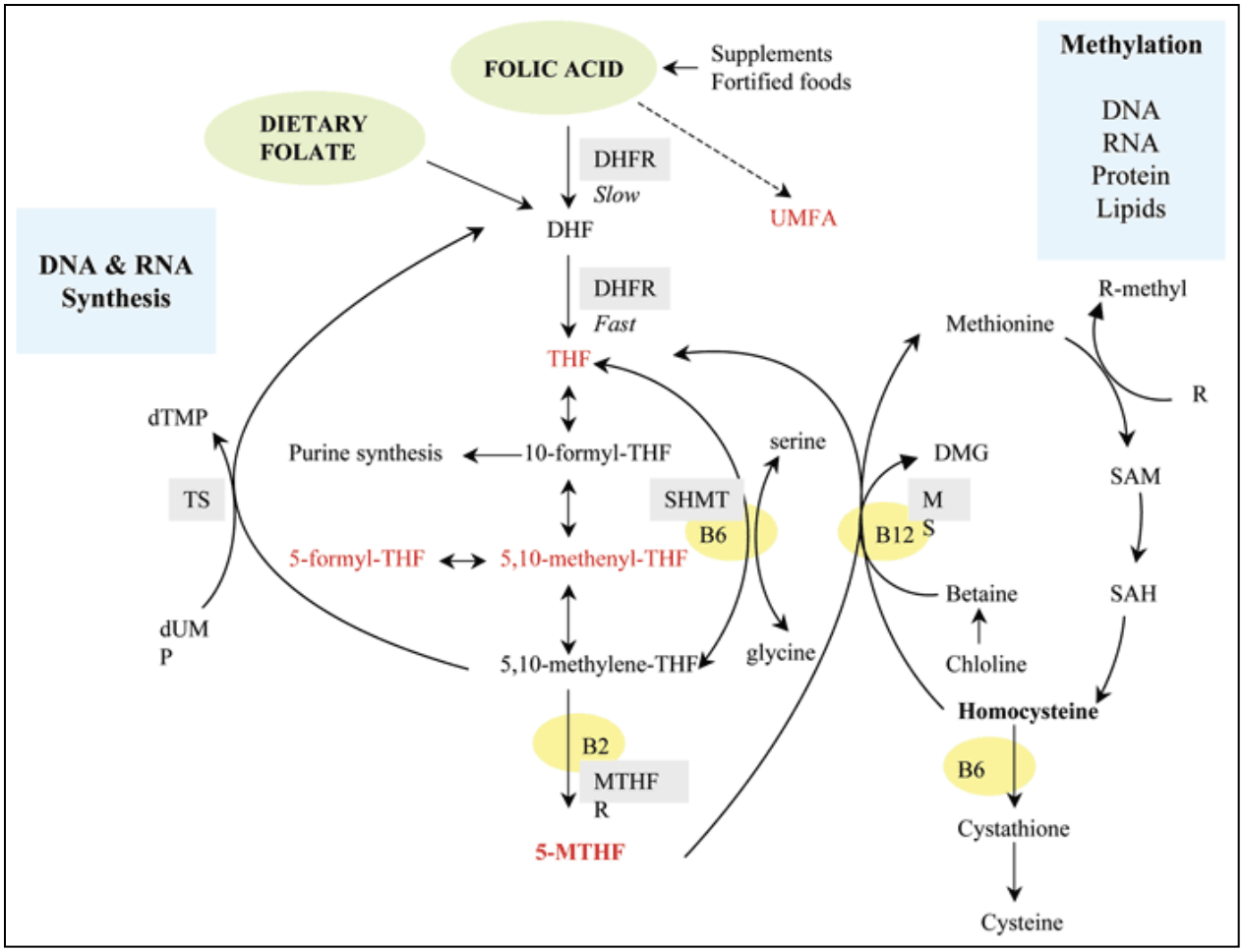

Figure 1-1: Folate Metabolism [14]

A polyglutamyl form of the tetrahydrofolate (THF) group is the main factor involved in one-carbon metabolism; this acceptor molecule is 5-methylenetetrahydrofolate (5-methyl THF) [15]. The most crucial step in the pathway to provide the 3 carbon serine (major carbon source) is the conversion of THF to 5, 10-methylene THF [15]. Serine uses this one-carbon unit to form 5, 10-methylene-THF and glycine from THF via serine hydroxymethyltransferase (SHMT). The enzyme responsible for the irreversible conversion of 5, 10 methylene-THF to 5-methyl THF is methylenehydrofolate reductase (MTHFR). The N-5 methyl group of 5-methyl THF can only be used metabolically for transfer to homocysteine, which results in the regeneration of methionine; in the methionine synthase reaction, demethylation of the 5-methyl THF occurs and this methyl

group is sequentially transferred to the vitamin B-12 coenzyme before homocysteine, thus forming methionine [15]. The produced methionine is then broken down to the methyl donor group: s-adenosylmethionine (SAM), as demethylation continues, s-adenosyl-L-homocysteine (SAH) is converted into homocysteine. Furthermore, the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP) via the enzyme thymidylate synthase (TS) occurs during the pyrimidine biosynthesis pathway [16]. The most important functions involved in folate metabolism are: the transfer of one-carbon in the methionine cycle, the synthesis of purines (adenine and guanine) and the DNA methylation reactions. These reactions are a required to maintain the integrity of genetic material and sustain DNA proliferation. However this pathway can be altered when folate status is compromised and can lead to harmful epigenetic changes.

When folate status is compromised, several reactions in one-carbon metabolism are affected and can lead to negative consequences. The regulation of this pathway is tightly controlled by the synthesis of methyl groups and other one-carbon units; folate and relevant coenzymes are categorized between mitochondria and cytosol [16]. The inhibition of MTHFR by SAM can suppress the production of 5-methyl-THF; therefore the presence of excess methyl groups can further the de novo biosynthesis of methyl groups [16]. Inadequate folate status during severe deficiency can impair one-carbon metabolism, however since 5-methyl and 5-formyl-THF play the role of SHMT inhibitors marginal deficiency may only effect the carbon flux marginally due to their effects being diminished [16]. In a low folate status condition, the regulation of SAM would be disrupted, the concentration of SAH would increase due to the failure of methyl group synthesis and homocysteine re-methylation [17].

E. Neurodegenerative Diseases

As stated earlier, inadequate folate intake during pregnancy increases the risk of abnormalities of the fetus. Folate deficiency has also been associated with neurodegenerative conditions, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and stroke in the elderly [18]. Folate deficiency alters SAM- dependent histone methylation. Compromised histone methylation has been associated with impaired learning and memory, intellectual disability, autism, and neurodegeneration [18]. A rise in homocysteine serum concentration and a decreased SAM/SAH ratio can impair cerebral function and interfere with cognitive function leading to dementia and Alzheimer's disease [19].

Alzheimer's is a progressive neurodegenerative disease associated with brain shrinkage and loss of neurons, leading to loss of brain function. Alzheimer's is characterized by loss of memory and language, irritability, confusion, dementia, aggressive moods and loss of physiological conditions [20]. A compromise in one-carbon metabolism can accompany and may be the possible cause of neurodegeneration of Alzheimer's Diseases [18]. In individuals with Alzheimer's disease, SAH levels are elevated and they competitively inhibit SAM-dependent reactions, which in turn reduces methylation reactions [18].

Elevated homocysteine levels have also been reported to cause cognitive decline and depression in patients with Parkinson's disease [21]. Parkinson's disease is a progressive nervous system disease that impairs movement. Homocysteine has the capability to exert neurotoxic effects which can decrease the movement and function on neural cells; elevated levels of homocysteine may increase the risk of Parkinson's disease by having a direct effect on dopaminergic neurons [21].

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disease which does not have an effective treatment or cure. It is characterized by neuron loss, psychiatric disturbances and dementia [22]. Higher plasma levels of homocysteine have been observed in patients with HD. Due to the regulatory effects of folic acid on homocysteine metabolism, it has been suggested that folic acid supplementation maybe helpful in HD [23]. However it has not yet been established that folic acid supplementation can provide any type of therapeutic effects in patients with HD.

The adverse effects of folate deficiency and elevated homocysteine levels on the developing central nervous system suggests that the alteration in one-carbon metabolism and homocysteine levels may play a role in the pathogenesis of age-related neurodegenerative diseases [24]. Homocysteine can damage, kill and promote the degeneration of neurons by impairing DNA repair; it has been suggested that elevated levels of homocysteine may increase the vulnerability of the CNS to neurodegenerative diseases [24].

F. mTOR Pathway

The mammalian target of rapamycin (mTOR) pathway responds to several environmental cues and integrates these signals to regulate cellular growth and homeostasis [25]. The mTOR pathway impacts most major cellular functions due to its regulation of basic cell functions of growth and proliferation [25]. The deregulation and/or alteration of this pathway occur in many human diseases, such as cancer, obesity type 2 diabetes and neurodegenerative diseases. mTOR is a serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K) family; it interacts with proteins to form two specific multi- complexes mTORC1 and mTORC2 [25].

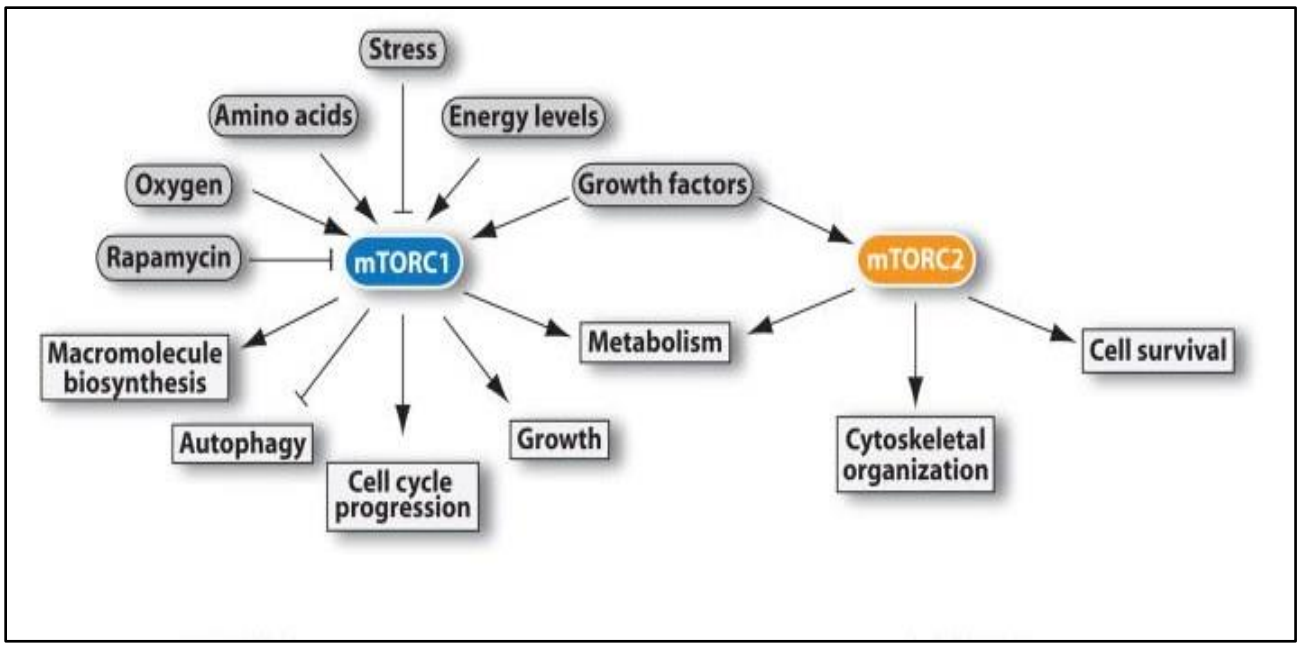

Figure 1-2: Overview of mTORC1 and mTORC2 [25]

The mTORC1 complex integrates inputs from intra and extra cellular environmental cues (stress, amino acids, energy status, and growth factors) to regulate processes of protein and lipid synthesis, energy metabolism, development and autophagy mainly [25]. The mTORC2 responds to growth factor signals to regulate cell survival and migration [26]. Both of the mTOR complexes have different sensitivities to rapamycin, along with different signaling of upstream input and downstream output [25]. The mTOR signaling network/pathway can be seen in the figure below:

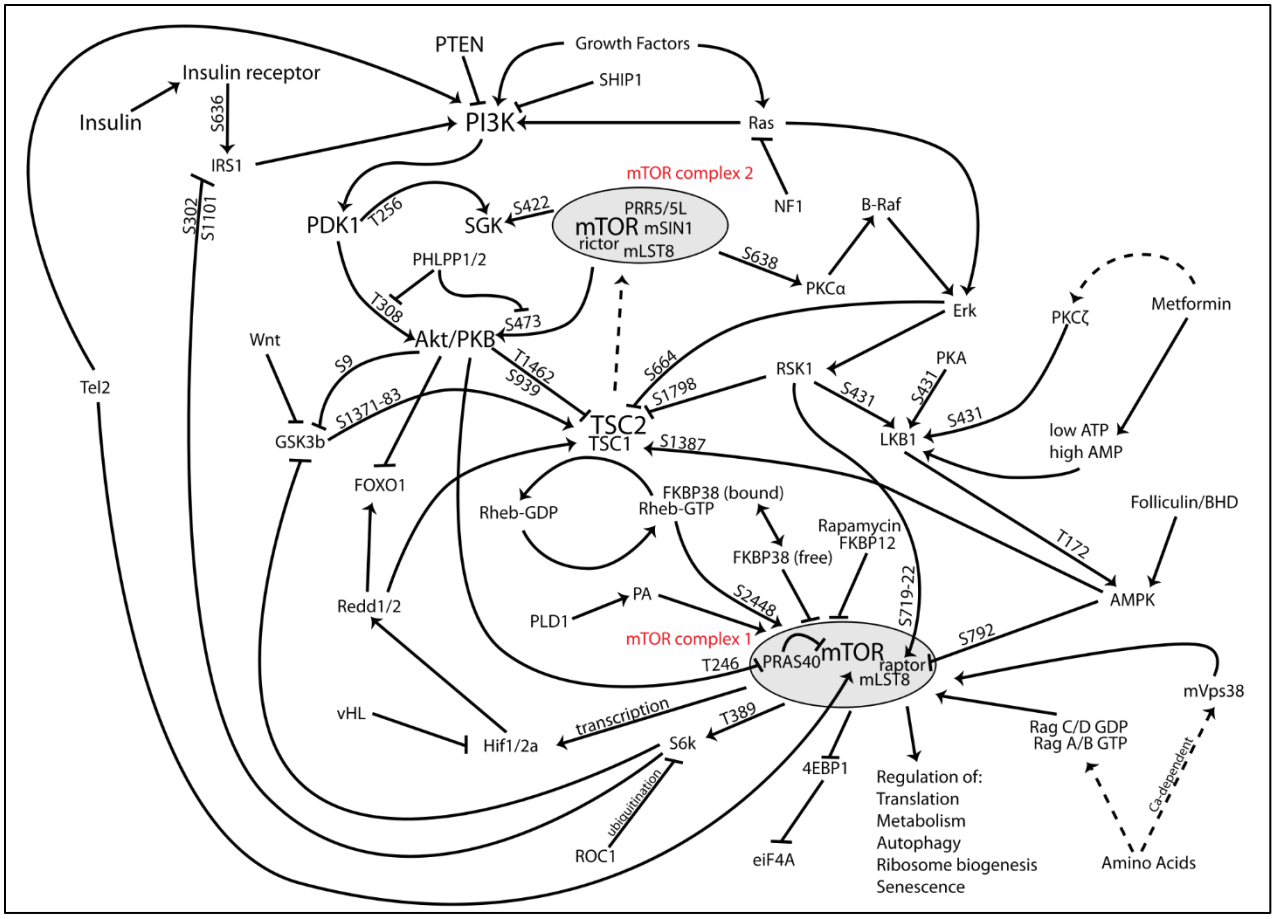

Figure 1-3: mTOR signaling Network [28]

The mTOR pathway responds to several environmental cues to impact most major cellular functions; basic cell functions of growth and proliferation. mTORC1 is activated by the PI3K and AKT pathways and mTORC2 is activated via signaling from the RAS and RAF (protein kinases) pathways [26]. As seen in the diagram: mTORC1 is composed of mTOR, PRAS40, mLST8 and raptor (rapamycin-sensitive); mTORC2 is composed of mTOR, PRR5/5L, mSIN1, mLST8 and rictor (rapamycin-insensitive) [28]. TSC1/2 functions as a GTP-ase activating protein for the Ras homolog enriched in the brain- Rheb; it acts as a key upstream regulator of mTORC1 to activate kinase activity and it transmits signals that affect mTORC1 (such as insulin and insulin-like growth factors) [28].

mTORC1 directly targets two translational initiation factors, 4E-BP1 and S6K1 by phosphorylating them to promote protein synthesis. The phosphorylation of S6K1 mediates the phosphorylation of and activates the 40S ribosomal protein S6 which in turn enhances translation of mRNA [29]. The phosphorylation of 4E-BP1 prevents it from binding to eIF4E (cap-binding protein), leading it to form the eIF4E complex required to mediate cap dependent translation [25, 29]. Growth factors activate mTORC1 indirectly by suppressing TSC1/2 function through phosphorylation of TSC2 by PI3K/AKT/mTOR pathway; AKT signals phosphorylates and causes the dissociation from raptor [25, 30].

By contrast the phosphorylation of TSC2 by AMPK due to low energy levels results in the activation of TSC1/2 thus inhibiting mTORC1 signaling and stimulating autophagy [25]. AMPK is a protein kinase which senses intracellular energy and becomes activated as a response to low nutrient availability and ATP depletion. REDD 1 is an upstream repressor of mTORC1 and is induced by stress, hypoxia, serum and nutrient deprivation. Although still unclear, it has been hypothesized that REDD 1 acts to promote the GAP activity of TSC2 toward Rheb resulting in the accumulation of Rheb-GDP and subsequent repression of mTORC1 signaling [26].

mTORC2 activates AKT via phosphorylation of the Ser473 form of AKT to indirectly regulate further activation of AKT [31]. This activation of AKT via mTORC2 signals positive feedback and could thereby indirectly activate mTORC1 [25, 31, 32]. Inositol polyphosphate multikinase (IPMK) is kinase that acts as a physiologic mTOR cofactor; it regulates nutrient amino acid signaling to mTORC1 [33]. IPMK uses its terminal amino acid sequence to stabilize the binding of raptor to mTOR in the mTORC1 complex [33].

G.mTOR and Neurodegenerative Diseases

Neurodegeneration is a condition in which the neurons of the brain lose structure and function, eventually leading to cell death. Alzheimer's, Parkinson's and Huntington's disease are characterized by the accumulation of misfolded/toxic proteins and related neuronal death. As the mTOR pathway is involved in several pathways of programmed cell death (autophagy, apoptosis and necroptosis) it is implicated to be involved in the pathophysiology of neurodegenerative diseases [34]. The mTOR signaling pathway plays in important role memory formation, fear, and loss of cognition; it may be required in the hippocampus for synaptic plasticity and memory formation [35]. Disruption of mTOR signaling can lead to the prevention of long-term potentiation and memory retention [35].

mTOR activation has been considered as a contributor to the progression of AD however the level of mTOR activity that is beneficial or detrimental in this condition has not been determined yet [35]. In mice, the survival of newborn neurons and dendritic density have been compromised by Beta-Amyloid (Aβ) due to the loss of AKT, mTOR, and p70S6K activity [36]. mTOR has been shown necessary for the formation of long-term memory in the amygdala; loss of mTOR function can impair memory consolidation [36]. Studies have shown that the activation of p70S6K has been associated with formation of hyper-phosphorylated tau and neurofibrillary accumulation. Aβ can be toxic to cells in brains of patients with AD, it can phosphorylate and activate mTOR and p70S6K in neuroblastoma cells and lymphocytes [35]. In murine models it has been found that the up-regulation of mTOR activity has been associated with long-term potentiation and synaptic plasticity [37]. Retinoblastoma tumor suppressor (RB1) inducible Coiled-Coil 1 (RB1CC1) is necessary for neurite growth maintenance of mTOR signaling. If there is an insufficiency of RB1CC1 expression, it can result in mTOR activity inhibition, and

thereby cause neuronal atrophy in patients with AD [37, 38]. Overall, there is growing evidence demonstrating that the Aβ exerts its toxicity via the mTOR pathway, further supporting the idea that reducing mTOR signaling in AD may be a valid therapeutic approach.

The level of mTOR signaling that can benefit or harm the condition of PD has also not been defined. It has been suggested that the inhibition of mTOR activity may be detrimental in PD [38]. Studies have shown that the expression of REDD1 is increased in Parkinson's brains (animal models), leading to the death of dopaminergic neurons [38]. Along with this, if the downstream effector, 4E-BP1 is activated by leucine-rich repeat kinase 2 (LRRK2), it can alter protein translation resulting in the loss of dopaminergic neurons [38, 39]. However there are studies that show mTOR inactivation may preserve dopaminergic neurons. Animal models using rapamycin as a possible therapeutic approach show that the signaling pathway of AKT maybe providing neuronal protection [40]. A decrease was observed in the loss of dopaminergic neurons due to the inhibition of mTOR signaling as a result of autophagy pathway activation [38, 41]. Animal models using rapamycin as a possible therapeutic approach show that the signaling pathway of AKT maybe providing neuronal protection. It was believed that the inhibition of mTOR signaling was a result of autophagy pathway activation [41].

In HD, it has been found that the inhibition of mTOR pathway via autophagy promoting factors may be a possible treatment. Autophagy is responsible for clearing intracellular huntingtin protein aggregates that can lead to neuronal degeneration therefore considered important [42]. In fly and murine models, it has been found that rapamycin inhibits mTOR activity by increasing the autophagic clearance of proteins with long polyglutamine or polyalanine expansions, thereby attenuating huntingtin accumulation and cell death in HD [42, 43]. However in some experimental models of HD, the inhibition of both mTOR complexes may

be needed to affect autophagy and the accumulation of huntingtin aggregates [42]. Studies show that a decrease in activity of p70S6K can protect against early decline in motor skills without having an effect on huntingtin protein levels [42]. Another study observed that the DNA damage protein 34 (GADD34) may be required for mTOR to provide neuroprotection in HD. GADD34 function leads to dephosphorylation of TSC2 and induction of autophagy, thereby increasing cell survival when it is overexpressed [42].

The failure to induce autophagy or the over-enhancement of the pathway can be an underlying aspect of certain brain pathologies [44]. Autophagy-related genes (ATGs) are key regulators of the autophagy process; mTOR acts as a regulator by suppressing the autophagy pathway under nutrient-rich conditions [44]. The inhibition of the TSC1/TSC2 as a result of the effects of growth factors and nutrient deprivation on the mTOR pathway causes activation of the mTORC1 complex, thereby inhibiting autophagy [44]. The first studies linking autophagy to the CNS were performed in animals in which essential regulatory genes (ATG5 and ATG7) were genetically inactivated [44]. It was found that inactivation of these regulatory genes resulted in spontaneous neurodegeneration, spontaneous accumulation of protein aggregates, neuronal loss and premature death [44]. An experimental study tested a Beclin 1(+/-) known-down crossed into an AD mouse model, to find that Aβ accumulation was promoted, suggesting that autophagy of Aβ aggregates may be inhibited in AD [45]. Another experiment, using two AD mouse models treated with rapamycin for 10-13 weeks was conducted to determine the role of autophagy in AD; rapamycin was found to promote autophagy to decrease Aβ accumulation [45, 46, 47]. These conflicting studies suggests there is a discrepancy in autophagy activity in the AD brain, therefore further studies must be conducted to elucidate this mechanism.

CHAPTER 2: SPECIFIC AIMS

The mTOR signaling pathway responds to several environmental cues and integrates these signals to regulate cell function, homeostasis and proliferation. The mTOR signaling pathway can impact multiple neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington's disease. mTOR signaling can affect the early development of cells through stem cell proliferation and differentiation as well as the end stages of cellular function leading to autophagy and apoptosis. It is important to understand and elucidate the mechanism by which folate deficiency has an effect on the mTOR signaling network in brain development and cognitive decline. It becomes crucial to understand how folate deficiency causes the pathway to become deregulated or over-enhanced to negatively impact brain development, cognition and memory. We hypothesize that dietary folate restriction alters the mTOR signaling network in the brain to provide protection against the onset and progression of neurodegenerative diseases.

Specific Aim 1:

To investigate if folate deficiency provides a protective role against factors contributing to neurodegenerative diseases in mice.

Specific Aim 2:

To examine the impact of folate deficiency within the mTOR signaling pathway to elucidate the signaling mechanisms of the substrates.

CHAPTER 3: METHODS

Animals

The experiments were performed in young adult (6 month), wild-type male C57BL/6 specific pathogen-free mice in accordance with NIH guidelines for the use and care of laboratory animals. The Wayne State University Animal Investigation Committee approved the animal protocol. The mice were maintained on a 12-hr light/dark cycle and fed standard mouse chow and water ad libitum. At 12 weeks of age, the mice were randomly assigned to two dietary groups and were fed AIN93G-purified isoenergetic diets (Dyets, Inc., Lehigh Valley, PA). Diets were stored at –20°C. 1% succinyl sulfathiazole was added to all diets. The control group received a folate adequate diet containing 2 mg/kg folic acid. The experimental group received a folate-deficient diet containing 0 mg/kg folic acid. The animals' food intake and body weights were monitored twice weekly to monitor for signs of toxicity and the experimental diets were continued for 8 weeks. At 24 weeks, mice were anesthetized in a CO_2 chamber and sacrificed by cervical dislocation. Harvested brain was flash frozen and stored in liquid nitrogen [4].

Whole Cell Extraction

Whole cell extraction was performed per standard protocol. 100 mg of brain tissue samples were extracted using a homogenizer. During the homogenizing process various, detergents were used: Igepal, Sodium deoxycholate, SDS, PBS, mainly to lyse the cells, denature some of the protein structures (but not fully damage the proteins), and to place a negative charge on the protein for it to be linearized. RIPA buffers were added to the brain tissue, it was homogenized, and thereafter the proteases were added to ensure protein solubility to inhibit any damages that may have been caused by the denaturing agents. Various spins were conducted as

protocol demands, to separate pellet from supernatant. The supernatant was the collected very carefully in a separate tube and put into -80ºC until ready to be used.

Western Blot Analysis

Western blot analysis was performed using 200 µg of crude nuclear extract isolated form brain tissue per standard protocol. To ensure the correct amount of protein concentration was used from each sample, the Bradford Assay was performed according to standard protocol and amounts were determined through normalization. Samples were then subjected to polyacrylamide gel electrophoresis (SDS-PAGE). Upon completion of SDS-PAGE, the region containing the protein(s) of interest was excised and prepared for western blot analysis while the remaining portion of the gel was stained with GelCode®Blue Stain Reagent (Pierce Biotechnology, Rockford, IL) to ensure equal protein loading. . Western blot analysis was conducted by using affinity purified polyclonal antisera developed against mouse target proteins. As an internal control for protein loading, membranes were reprobed with anti-Lamin B antibody (Santa Cruz Biotechnology, santa Cruz, CA). The bands were visualized and quantified using a ChemiImager® System (Bio-Rad, Hercules, CA) after incubation in SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Data are expressed as the integrated density value (I.D.V.) of the band per µg of protein loaded [4].

Statistical Analysis

Statistical significance was determined using an “unpaired t-test”. P values less than 0.05 were considered statistically significant.

CHAPTER 4: FIGURES

Figure 4-1: Experimental Design

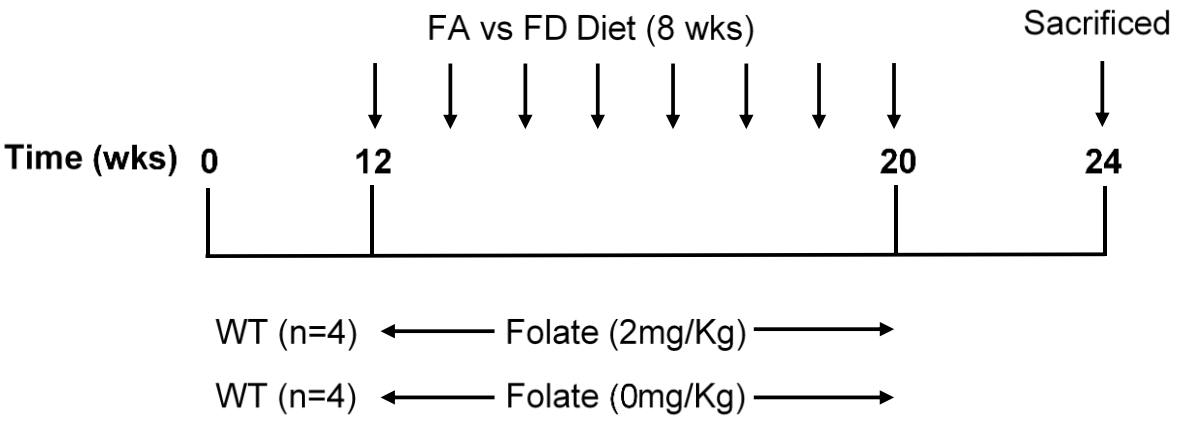

Figure 4-1Experimental Design: The control mice were given a folate adequate diet (2mg/kg body weight) and the experimental mice were given folate deficient diet (0mg/kg body weight). The animals' food intake and body weights were monitored twice weekly to monitor for signs of toxicity and the experimental diets were continued for 8 weeks. Mice were then sacrificed for analysis at 24 weeks.

Figure 4-2: Effect of folate adequate vs. folate restricted diet on protein levels of β-actin in C57BL/6 mice

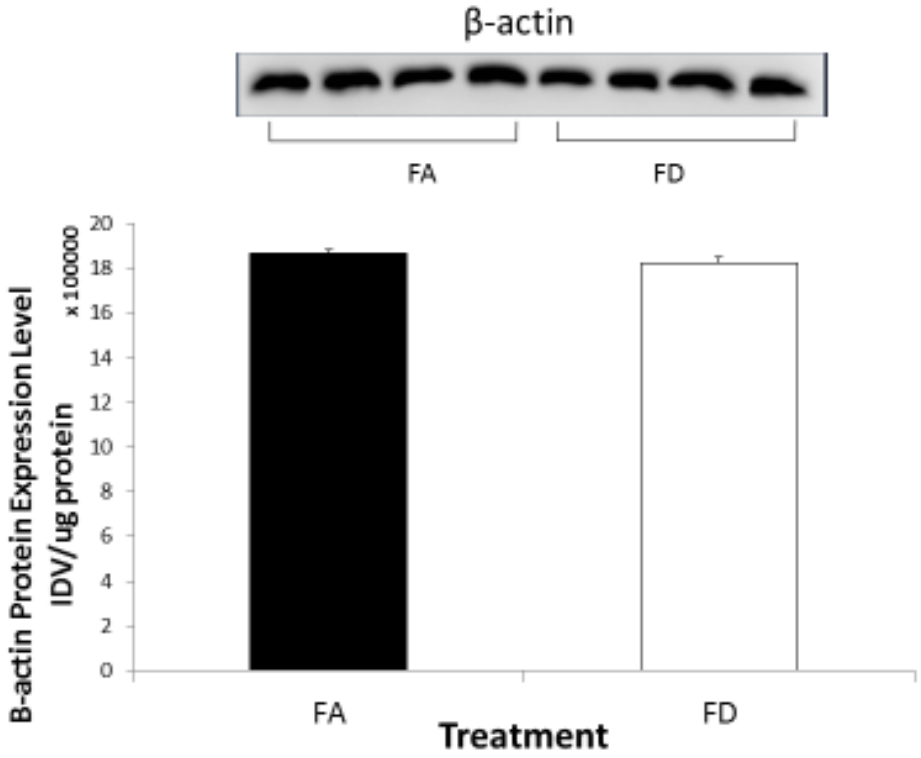

Figure 4.2 Effect of folate adequate vs. folate restricted diet on protein levels of β-actin in C57BL/6 mice: In the above figure, an analysis of β-actin protein levels in brain of young wildtype mice is shown. Experimental mice were given folate deficient diets as described previously while control mice were given a folate adequate diet. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 4 mice of control group and 4 mice of experimental group. Values with an asterisk superscript indicates significant difference ($p<0.05$). Levels of β-actin were found to be statistically insignificant between control and experimental groups. T-test = 0.260963

Figure 4-3: Effect of folate adequate vs. folate restricted diet on protein levels of Beclin in C57BL/6 mice

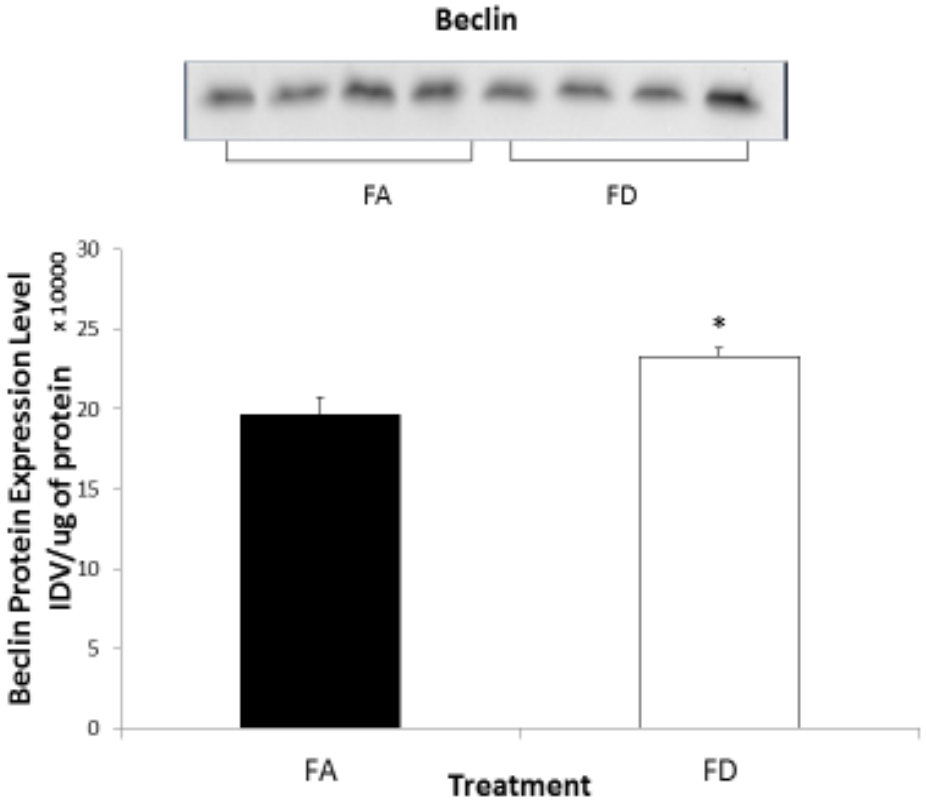

Figure 4.3 Effect of folate adequate vs. folate restricted diet on protein levels of Beclin in C57BL/6 mice: In the above figure, an analysis of Beclin protein levels in brain of young wildtype mice is shown. Experimental mice were given folate deficient diets as described previously while control mice were given a folate adequate diet. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 4 mice of control group and 4 mice of experimental group. Values with an asterisk superscript indicates significant difference ($p<0.05$). Levels of Beclin show significant difference comparing control vs. experimental diets. T-test = 0.025006

Figure 4-4: Effect of folate adequate vs. folate restricted diet on protein levels of ATG7 in C57BL/6 mice

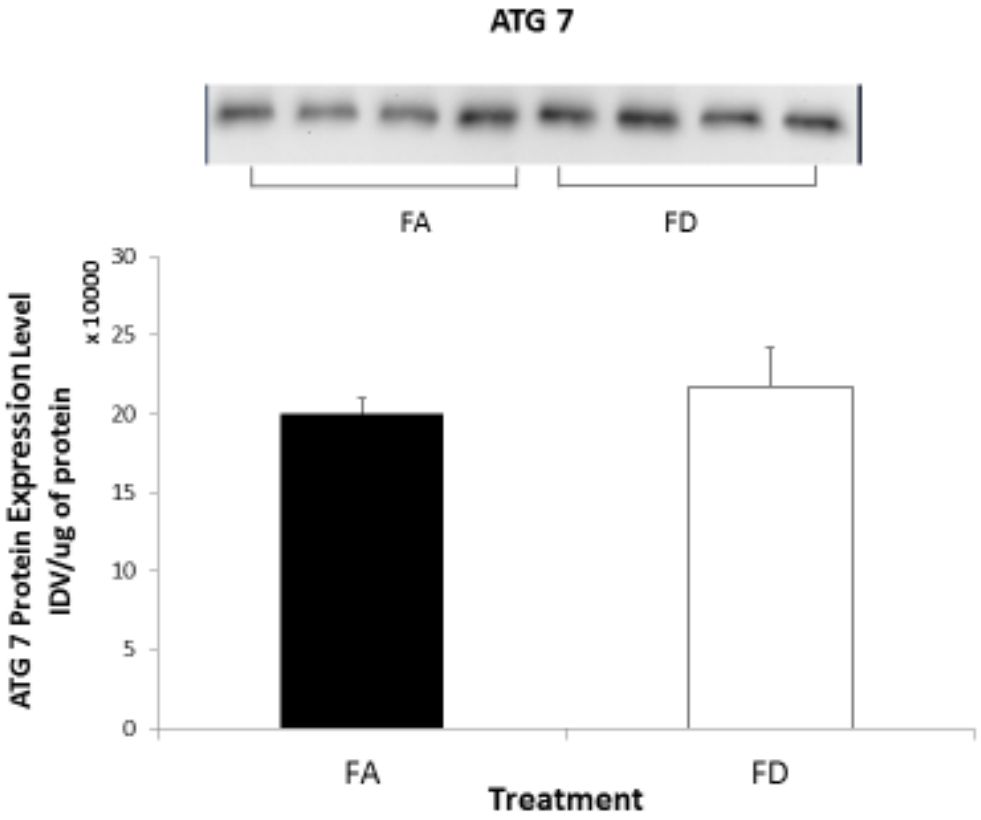

Figure 4.4 Effect of folate adequate vs. folate restricted diet on protein levels of ATG7 in mice: In the above figure, an analysis of ATG7 protein levels in brain of young wildtype mice is shown. Experimental mice were given folate deficient diets as described previously while control mice were given a folate adequate diet. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 4 mice of control group and 4 mice of experimental group. Values with an asterisk superscript indicates significant difference ($p<0.05$). Levels of ATG7 show no significant difference comparing control vs. experimental diets.T-test = 0.471038

Figure 4-5: Effect of folate adequate vs. folate restricted diet on protein levels of LC3 in C57BL/6 mice.

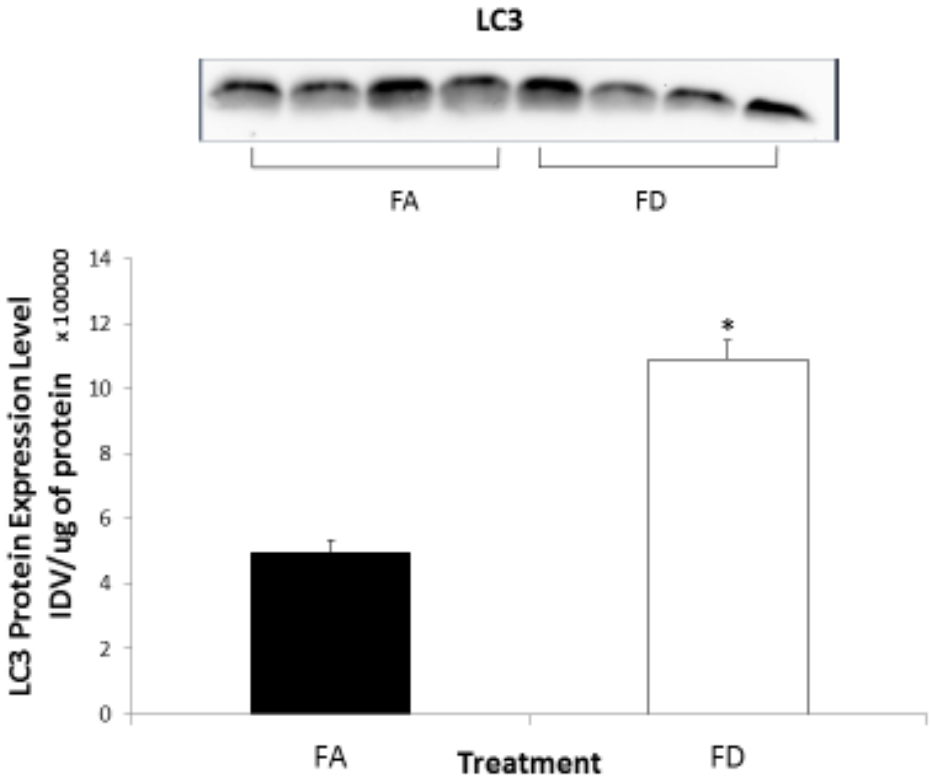

Figure 4.5 Effect of folate adequate vs. folate restricted diet on protein levels of LC3 in C57BL/6 mice: In the above figure, an analysis of LC3 protein levels in brain of young wildtype mice is shown. Experimental mice were given folate deficient diets as described previously while control mice were given a folate adequate diet. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 4 mice of control group and 4 mice of experimental group. Values with an asterisk superscript indicates significant difference ($p<0.05$). Levels of LC3 show significant difference comparing control vs. experimental diets. T-test = 0.00054

Figure 4-6: Effect of folate adequate vs. folate restricted diet on protein levels of IPMK in C57BL/6 mice

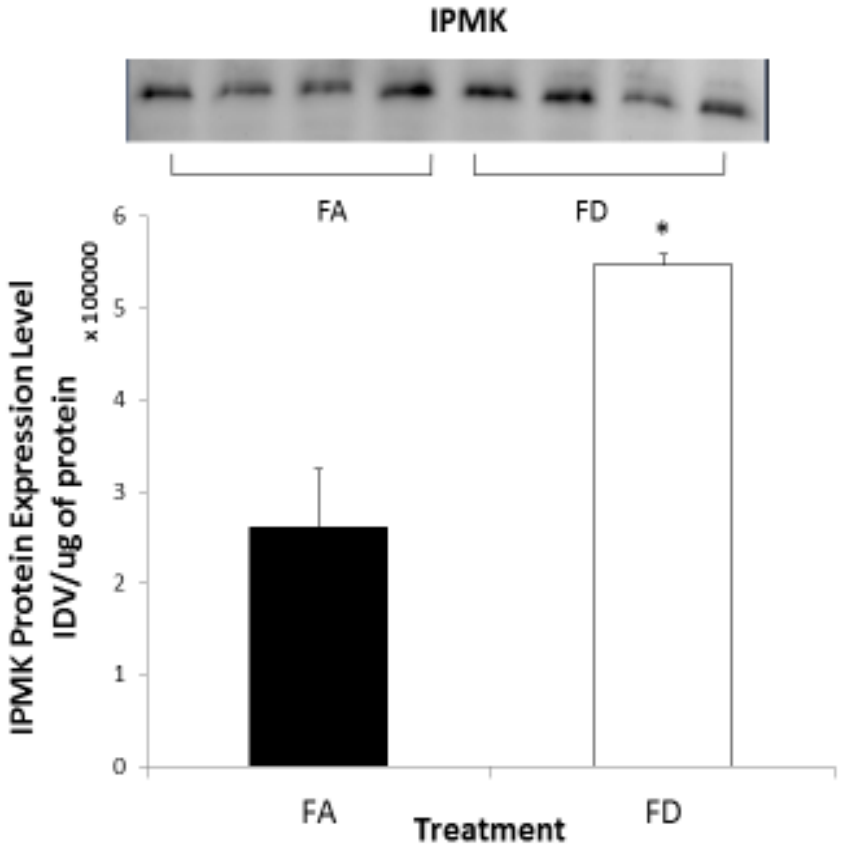

Figure 4.6 Effect of folate adequate vs. folate restricted diet on protein levels of IPMK in C57BL/6 mice: In the above figure, an analysis of IPMK protein levels in brain of young wildtype mice is shown. Experimental mice were given folate deficient diets as described previously while control mice were given a folate adequate diet. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 4 mice of control group and 4 mice of experimental group. Values with an asterisk superscript indicates significant difference ($p<0.05$). Levels of IPMK show significant difference comparing control vs. experimental diets.
T-test = 0.005604002

Figure 4-7: Effect of folate adequate vs. folate restricted diet on protein levels of REDD-1 in C57BL/6 mice

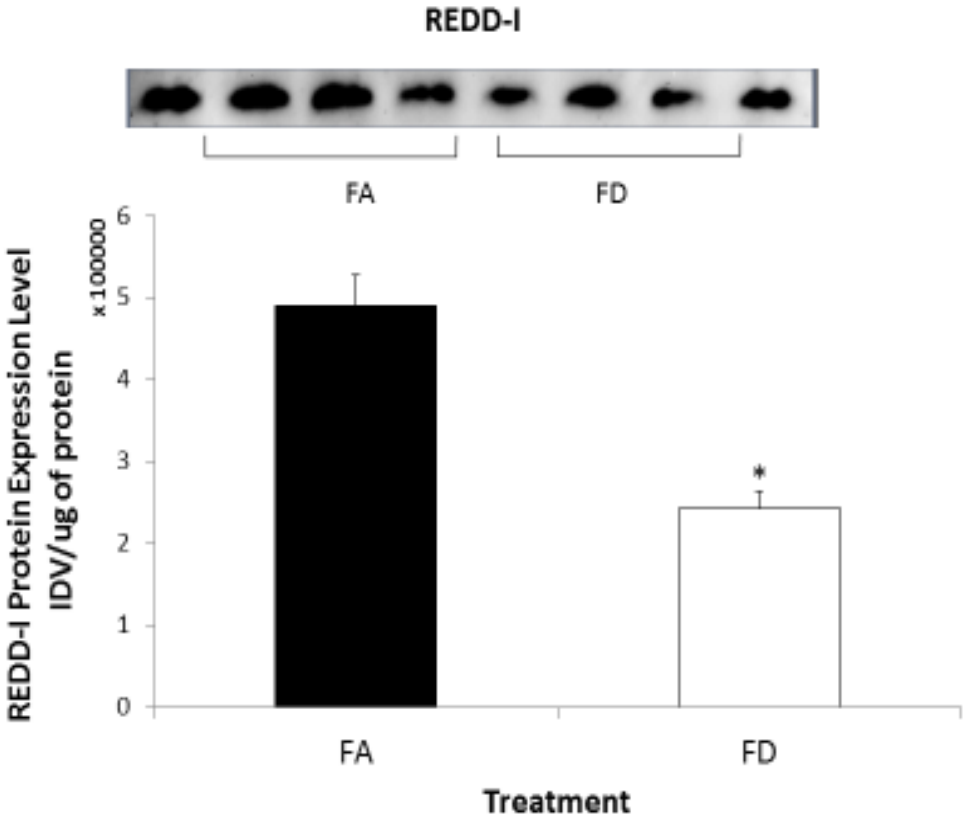

Figure 4.7 Effect of folate adequate vs. folate restricted diet on protein levels of REDD-1 in C57BL/6 mice: In the above figure, an analysis of REDD-1 protein levels in brain of young wildtype mice is shown. Experimental mice were given folate deficient diets as described previously while control mice were given a folate adequate diet. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 4 mice of control group and 4 mice of experimental group. Values with an asterisk superscript indicates significant difference ($p<0.05$). Levels of REDD-1 show significant difference comparing control vs. experimental diets.
T-test = 0.002287696

Figure 4-8: Effect of folate adequate vs. folate restricted diet on protein levels of p-AMPK in C57BL/6 mice

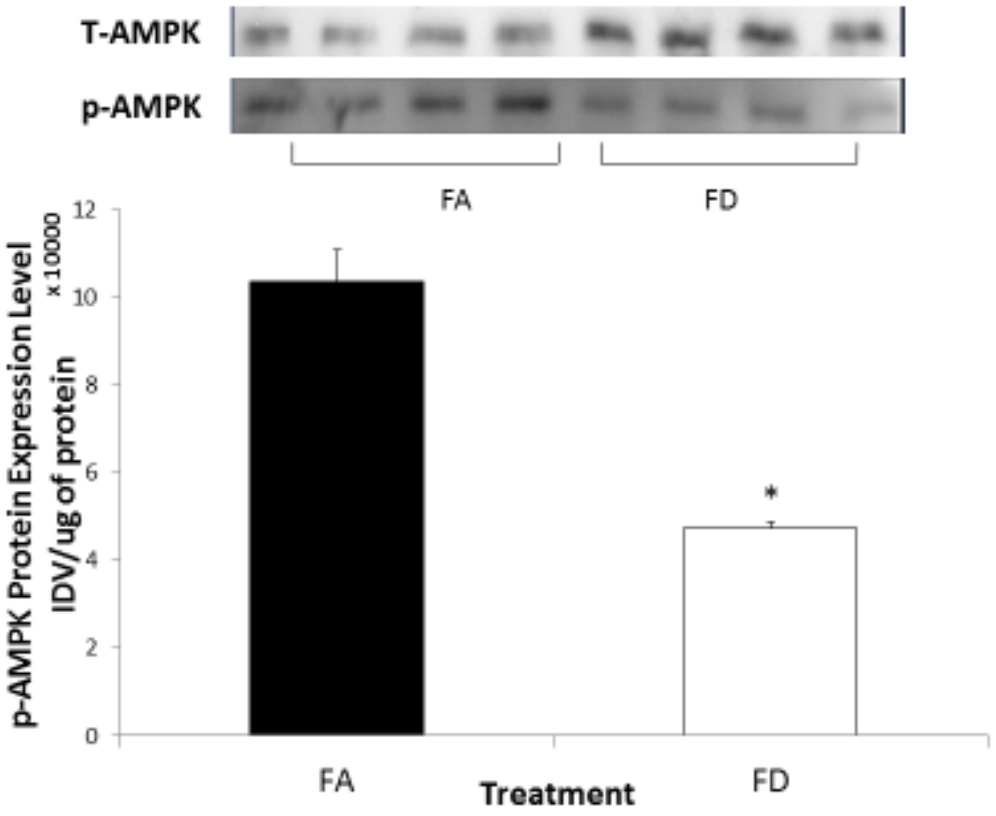

Figure 4.8 Effect of folate adequate vs. folate restricted diet on protein levels of p-AMPK in C57BL/6 mice: In the above figure, an analysis of p-AMPK protein levels in brain of young wildtype mice is shown. Experimental mice were given folate deficient diets as described previously while control mice were given a folate adequate diet. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 4 mice of control group and 4 mice of experimental group. Values with an asterisk superscript indicates significant difference ($p<0.05$). Levels of p-AMPK show significant difference comparing control vs. experimental diets.
T-test= 0.000874041

Figure 4-9: Effect of folate adequate diet vs. folate restricted on protein levels of p-AKT in C57BL/6 mice

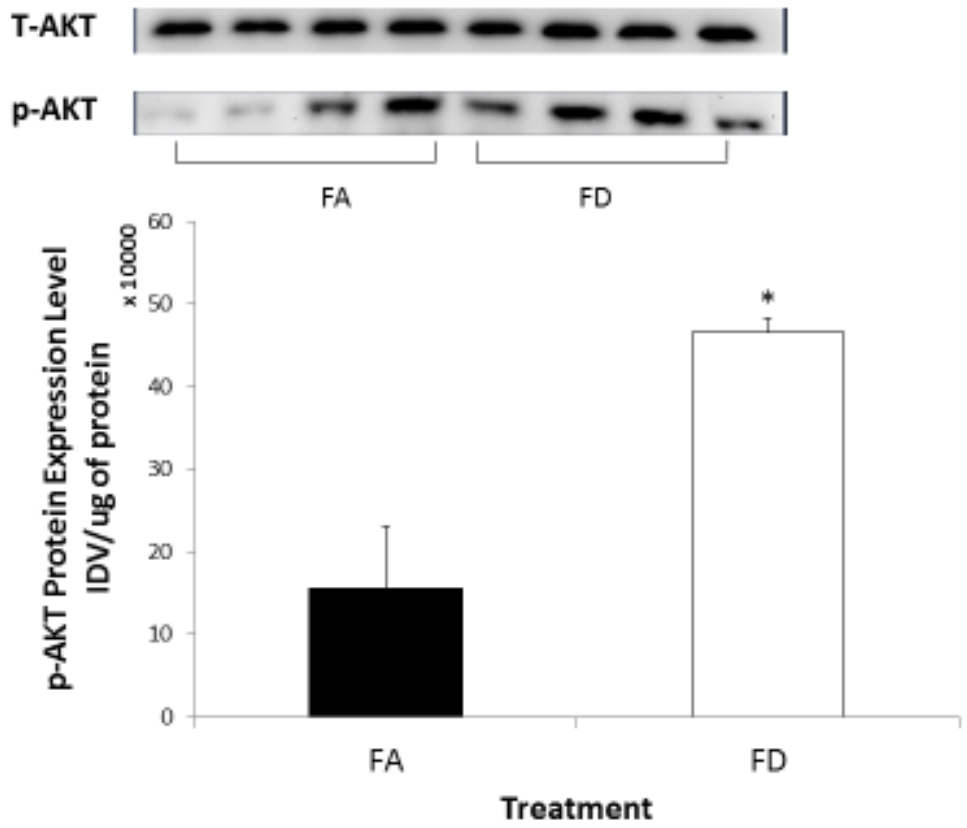

Figure 4.9 Effect of folate adequate diet vs. folate restricted on protein levels of p-AKT in C57BL/6 mice: In the above figure, an analysis of p-AKT protein levels in brain of young wildtype mice is shown. Experimental mice were given folate deficient diets as described previously while control mice were given a folate adequate diet. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 4 mice of control group and 4 mice of experimental group. Values with an asterisk superscript indicates significant difference ($p<0.05$). Levels of p-AKT show significant difference comparing control vs. experimental diets. T-test= 0.007345.

Figure 4-10: Effect of folate adequate vs. folate restricted diet on protein levels of p-S6K in C57BL/6 mice

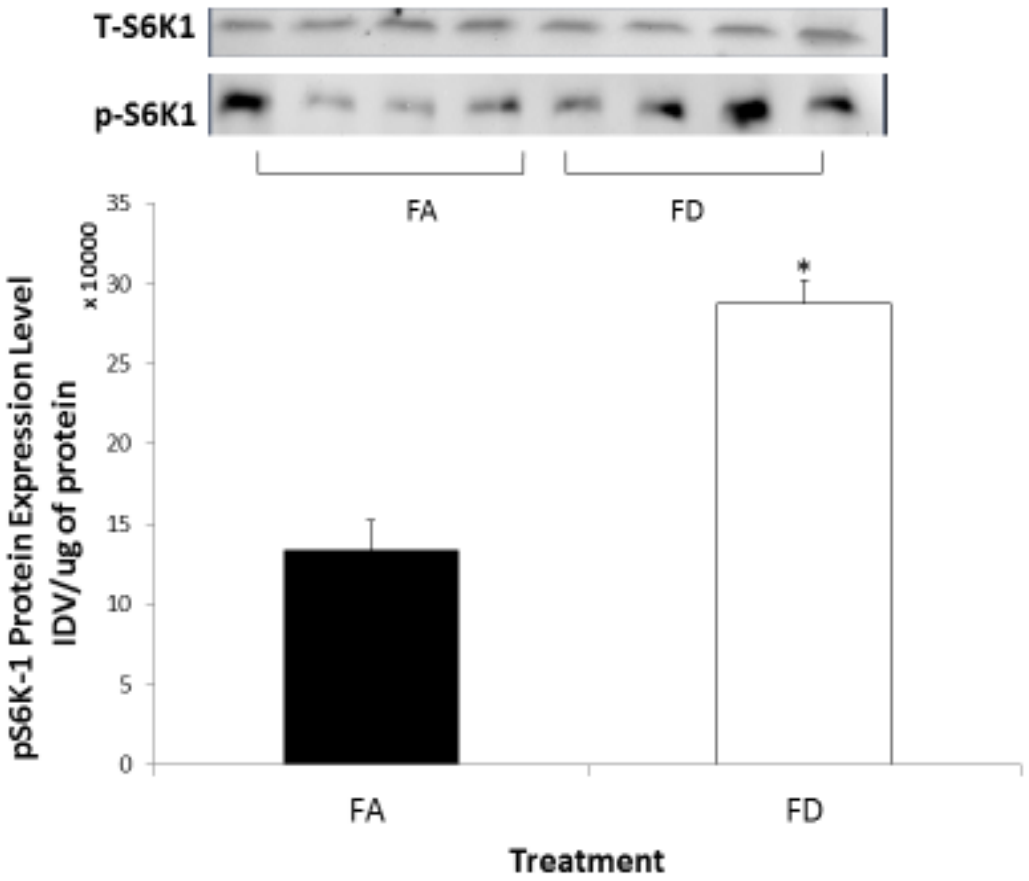

Figure 4.10 Effect of folate adequate vs. folate restricted diet on protein levels of p-S6K in C57BL/6 mice: In the above figure, an analysis of p-S6K protein levels in brain of young wildtype mice is shown. Experimental mice were given folate deficient diets as described previously while control mice were given a folate adequate diet. The protein levels were quantified using Western Blot analysis. Values represent an average (standard error of the mean) of data obtained from 4 mice of control group and 4 mice of experimental group. Values with an asterisk superscript indicates significant difference ($p<0.05$). Levels of p-S6K show significant difference comparing control vs. experimental diets. T-test= 0.001465474.

CHAPTER 5: RESULTS

Folate deficiency has been associated with neurodegenerative conditions in the elderly; previous studies have not focused on the effect folate deficiency has on young adult mice. There have been studies that have observed the influence of rapamycin and the mTOR pathway in the pathologies of neurodegenerative diseases. The mTOR pathway is stimulated by various environmental cues and regulates cell processes based on these external cues. It is important to understand and elucidate the mechanism by which folate deficiency causes the mTOR pathway to become activated or inhibited to attenuate the onset or progression of pathologies characteristic of neurodegenerative diseases. An experimental design was prepared in our laboratory to investigate if folate deficiency provides a protective role against factors contributing to neurodegenerative diseases via the mTOR pathway. An experimental folate diet prepared by Harrison et al., was used by our laboratory to conduct this study. This diet was tested in adult C57/BL6 mice; the mice were separated in two groups: one control and one experimental group at 3-4 weeks of age. Each group was fed its respective diet ad libitum for 8 weeks and then sacrificed for analysis (**Figure 4-1**) as described in the materials and methods section. The samples were obtained from each animal by performing whole cell extraction as described in the materials and methods section.

To investigate the effect of the diet in the brain of these mice, a western blot protein analysis (as described in the methods section) was conducted. **Figure 4-2** represents a trial conducted for the control protein, β-Actin, as expected consistent protein expression is observed in each sample. Autophagy plays a key role in the pathology and progression of Alzheimer's, Parkinson's and Huntington's disease. Autophagy is a catabolic process used by cells to clear any damaged proteins and organelles to survive in changing environments. The next thee figures

focus on autophagy factors of the mTOR pathway. **Figure 4-3** displays the effect of folate deficiency on the protein Beclin1, a significant difference is observed between both control groups. Beclin1 plays a central role in autophagy and is also required for ATG5/ATG7 dependent and independent autophagy [48]. A higher level of signaling is observed in the FD mice, therefore autophagy is being promoted. The observed expression of Beclin 1 suggests that autophagy is not being inhibited, hence possibly providing protection against the accumulation of Aβ and huntingtin protein aggregates in Alzheimer's and Huntington's respectively. In **Figure 4-4**, the levels of ATG7 do not show a significant difference, however it can be observed that the FD mice have higher signaling outputs then the FA mice. ATG7 is an E1-like activating enzyme which activates ATG12 for its conjugation with ATG5 and ATG8. ATG7 is required for mitophagy which contributes to regulate mitochondrial quantity and quality by eliminating the mitochondria to a basal level to fulfill cellular energy requirements and prevent the excess production of ROS (reactive oxygen species) [49]. It modulates the activity of p53/TP53 to regulate cell cycle and survival during metabolic stress. ATG7 also plays a key role in the maintenance of axonal homeostasis and the prevention of axonal degeneration [49]. This finding suggests that the animals are responding to the FD diet and perhaps compensating to survive by attempting to inhibit mTOR activity at some level. If the animals were on the diet for a longer period of time, we may be able to see a more significant difference between FA and FD animals. LC3 is used as another autophagy marker due to its ability to be involved with inner and outer membrane of the autophagosome. LC3 is synthesized as pro-LC3, which is the processed by ATG4 into its cytosolic form, LC3-I. During autophagosome formation, ATG3 and ATG7 mediate the covalent linkage of LC3-I to phosphatidylethanolamine (PE), for it to be incorporated into autophagosome membranes. This process converts LC3-I into LC3-II

indicating autophagy activity [50]. **Figure 4-5** displays the expression of protein levels of LC3, a significant difference is observed between both animal groups. However in western blot analysis two bands of the LC3 protein must be detected two signify cleavage and this was not observed. LC3-I is covalently linking to phosphatidylethanolamine (PE) to be converted into LC3-II because there is an indication of autophagy occurrence however the level of conversion may be very low. This may also explain the insignificant expression levels of ATG7 observed in these animals. Nonetheless, the findings as related to autophagy factors suggest that there are protective measures being observed against neurodegeneration.

IPMK has been classified as a physiologic mTOR cofactor and it regulates nutrient amino acid signaling to mTORC1 [33]. IPMK mediates direct binding with mTOR through its unique amino terminus domain; it senses the level of amino acids present in the cell and activates mTOR [51]. **Figure 4-6** indicates that there is a significant difference in the expression of IPMK levels, the FD group exhibits much higher expression indicating mTOR activation. An accumulation of amino acids may be inducing this increased expression of IPMK. REDD-I is an upstream repressor of mTORC1 and is induced by stress, hypoxia, serum and nutrient deprivation. In **Figure 4-7** the levels of expression of the REDD-I protein are higher in the control group than that of the experimental group. Lower expression of REDD-I in the experimental group suggests that the folate deficient diet is not creating a hypoxic environment; perhaps mTOR activity is not de-regulated. In Parkinson's brains the increased expression of REDD-I has been proven to be detrimental as it leads to the death of dopaminergic neurons, therefore this finding suggests that the FD diet may be acting as a protective measure in PD. AMPK is a protein kinase which senses intracellular energy and becomes activated as a response to low nutrient availability and ATP depletion. AMPK is an important regulator of protein synthesis and energy (lipid and

carbohydrate) metabolism. The activation of AMPK inhibits the mTORC1 cascade by interacting with raptor and activating TSC proteins to inhibit Rheb; consequently decreasing the level of mTORC1. **Figure 4-8** displays lower p-AMPK levels in the experimental group. Indicating that AMP: ATP energy levels are sufficient, therefore potentially resulting in activation of the mTOR pathway. This finding correlates with the levels of protein of p-AKT and p-S6K expression observed in the FD diet animals.

mTORC1 is activated by the PI3K/AKT pathway; under normal conditions AKT is responsible for cell proliferation and endurance. **Figure 4-9** exhibits higher levels of p-AKT expression in the FD animals. AKT s473 phosphorylation is mTORC2 dependent. This implies that folate deficiency appears to increase mTORC2 activity as a compensatory mechanism to maintain insulin sensitivity and increase survival. The protein, p70S6K acts directly upon the mTORC1 complex and is responsible for increasing protein synthesis and cellular proliferation. mTOR and p70S6K pathways are important in the CNS as amino acids control cortical function through these pathways. Glutamate and leucine depend upon p70S6K to modulate synaptic signaling and food intake [36].**Figure 4-10** displays the effect of the folate deficient diet on the protein S6K-1, a significant difference is observed in which the FD group exhibits higher expression levels of S6K-1, thereby rendering mTORC1 active. Some studies have proven that hyperactive signaling of mTOR can have detrimental effects in AD patients, nonetheless there have been studies which have proven that complete mTOR inhibition can be harmful too.

CHAPTER 6: DISCUSSION

Folate deficiency has been studied extensively due to its association with chronic diseases, such as Alzheimer's disease, cancer and cardiovascular disease. In this study we focus on the effect folate deficiency has on the onset and progression of neurodegenerative diseases in relation to the mTOR pathway. In response to the FD diet we observed up-regulation of autophagy, however increased expression of p-S6K and p-AKT contrary to decreased REDD-I and increased IPMK expression suggested mTOR activation. Higher autophagy occurrence was indicated by increased expression of Beclin and LC3. Interestingly enough, even though IPMK levels suggested mTOR inhibition, another upstream effector p-AMPK suggested mTOR activation.

REDD-I, IPMK and AMPK are upstream effectors of the mTOR pathway. REDD-I is elevated during hypoxic conditions and nutrient deprivation, however the results indicate that folate deficiency is not creating a hypoxic environment therefore mTOR may not be effected by the expression of REDD-I. In Parkinson's brains the increased expression of REDD-I has been proven to be detrimental as it leads to the death of dopaminergic neurons, therefore this finding suggests that the FD diet may be acting as a protective measure in PD. IPMK expression is found to be elevated in the folate deficient mice, suggesting that mTOR activity is actually inhibited. One proposal for this behavior is that folate deficiency may be preventing protein synthesis by inhibiting mTORC1, which could subsequently lead to a buildup of amino acids. Leading to the over-expression of IPMK without stimulating mTORC1. Previous studies conducted in our laboratory with folate deficient mice have exhibited lower levels of amino acid and ATP. Phosphorylated AMPK acts as an energy sensor in the cell, inhibiting mTOR when energy is low. In nutrient deprivation conditions like folate deficiency, energy depletion is

expected and is expressed via p-AMPK levels. However in this study the expression of p-AMPK is interestingly found to be lower in the FD mice, indicating that ATP levels are sufficient, rendering mTORC1 active. The lower expression of p-AMPK observed in the folate deficient mice is an unexpected finding; perhaps since IPMK levels are higher there is a buildup of amino acids triggering sufficient ATP levels.

The expression of Beclin, ATG7 and LC3 is higher in the folate deficient group, indicating higher levels of autophagy. The failure to induce autophagy has been associated with brain pathologies, hence the up-regulation of autophagy exerted by the folate deficient diet may be acting as a protective measure. The levels of Beclin and LC3 were found to be significant, however LC3 expression did not provide the two bands for analysis, suggesting slow conversion of LC3-I to LC3-II , thus autophagy is occurring but at a slow rate. Even though ATG7 levels were not found to be significant the observed results still show that the levels are higher in the folate deficient mice. This expression may be related to the lower conversion rate of LC3-I to LC3-II. Autophagy causes the inhibition of the mTOR pathway, however the expression levels observed in p-S6K and p-AKT suggests that mTOR activation is occurring. p-S6K and p-AKT are both downstream effectors of the mTOR pathway. p-S6K is responsible for protein synthesis, cell proliferation and it increases the activity of glycogen synthase kinase, which increases glucose concentrations. We expected to observe a decrease in p-S6K levels due to the higher levels of autophagy observed. Possibly the lower level of REDD-I expression is relaying a message within the cell for S6K to not be phosphorylated since it is not in a hypoxic condition. This increase in p-S6K activity may be influencing p-AKT activity to be increased due to higher levels of glucose concentrations being sensed by the cell. Growth factors activate mTORC1 indirectly by suppressing TSC1/2 function through phosphorylation of TSC2 by

PI3K/AKT/mTOR pathway. In the FD animals, p-AKT may somehow be prevailing the signaling of REDD-I and IPMK to the TSC1/2 complex for mTOR activity to be inhibited via phosphorylation of TSC2.

Our experiments indicate that there has been modulation of the mTOR pathway in the brain of young adult mice fed a folate deficient mice. The observed results indicate that there may be an increase in autophagy that occurs in neuronal cells of mice treated with a folate deficient diet. On the contrary, a folate deficient diet seems to increase the flux of the mTOR pathway. The confounding results of this study suggests that more research must be done to fully understand this relationship. Perhaps a study conducted with the animals on the diet for a longer period of time may provide further insight into these findings.

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ABSTRACT

EFFECT OF FOLATE DEFICIENCY ON EXPRESSION OF PROTEINS ON THE mTOR SIGNALING PATHWAY IN THE BRAIN OF C57/BL6 MICE

by

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Nutrient-gene interactions can significantly impact several cellular processes in the human body by altering important molecular pathways. Nutrition can affect brain structure, function and development throughout the life cycle; it can have a profound effect on cognition ad mental health leading to neurodegenerative diseases. It is important to elucidate the mechanisms by which diet can effect signaling pathways in the brain. The alteration of the mTOR pathway has been implicated to be involved in the pathophysiology of neurodegenerative diseases. Due to the established role of folate playing a role in modulating the mTOR pathway, we decided to investigate how folate deficiency alters mTOR signaling in the brain. We hypothesized that dietary folate restriction alters the mTOR signaling network in the brain to provide protection against the onset and progression of neurodegenerative diseases. In response to the FD diet we observed higher autophagy occurrence however increased expression of p-S6K and p-AKT contrary to decreased REDD-I and increased IPMK expression suggested mTOR activation. Higher autophagy occurrence was indicated by increased expression of Beclin and LC3. Interestingly enough, even though IPMK levels suggested mTOR inhibition, another upstream effector p-AMPK suggested mTOR activation.

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