Gestational Diet Folic Acid Content and Development and Function of Food Intake Regulation in Rats

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Department of Nutritional Sciences
University of Toronto

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2018

ABSTRACT

High folic acid consumed during pregnancy promotes characteristics of metabolic syndrome in rat offspring. We hypothesized that folic acid alters the development of hypothalamic food intake pathways in utero, contributing to the metabolic phenotype of the offspring in later life. Pregnant Wistar dams were fed modified AIN-93G diets containing folic acid at: 0, 1.0 (control), 2.5, 5.0, or 10.0-fold of the recommended level. The folic acid content in the gestational diet was reflected in the expression of neuropeptide Y (NPY) neurons and body weight of the male offspring at birth. Low and high gestational folic acid led to higher food intake and weight gain in the offspring to 9 weeks post-weaning, consistent with decreased activity of pro-opiomelanocortin (POMC) neurons. The folic acid content in the gestational diet affects in utero development of hypothalamic feeding circuits and contributes to a phenotype that reflects characteristics of the metabolic syndrome in the offspring.
Acknowledgements

“And we know that in all things God works for the good of those who love him, who have been called according to His purpose.” – Romans 8:28

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Contributions

Neil Yang contributed to the study design, data collection, conducted the statistical analyses, interpreted the data and prepared the manuscripts and thesis.

Emanuela Pannia contributed to data collection and statistical analyses and critically reviewed the manuscript and thesis.

Dr. Diptendu Chatterjee contributed to the study design, data collection, provided technical assistance and advice, and reviewed the manuscript.

Dr. Ruslan Kubant contributed to the study design and data collection, provided technical assistance and advice, and reviewed the manuscript.

Mandy Ho and Rola Hammoud contributed to data collection.

Dr. G. Harvey Anderson provided laboratory resources, contributed to the study design and critically reviewed the manuscript and thesis.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AIN-93G</td>
<td>American Institute of Nutrition-93 growth</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate nucleus of the hypothalamus</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti related peptide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BHMT</td>
<td>Betaine homocysteine methyltransferase</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine-and-amphetamine-regulated transcript</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine phosphate guanine</td>
</tr>
<tr>
<td>DHF</td>
<td>Dihydrofolic acid</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolic acid reductase</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signaling-regulated kinases</td>
</tr>
<tr>
<td>FR</td>
<td>Folate receptor</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-aminobutyric acid-A</td>
</tr>
<tr>
<td>HV</td>
<td>High multivitamin</td>
</tr>
<tr>
<td>HVRF</td>
<td>High multivitamin with recommended folic acid</td>
</tr>
<tr>
<td>HMethyl</td>
<td>Recommended multivitamin with 10-fold folic acid, vitamin B12 and B6</td>
</tr>
<tr>
<td>HFol</td>
<td>Recommended multivitamin with 10-fold folic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>InsR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>JACK/STAT</td>
<td>Janus-activated kinase/signal transducer</td>
</tr>
<tr>
<td>Mash</td>
<td>Mammalian archaete-scute homologous gene</td>
</tr>
<tr>
<td>Mc4R</td>
<td>Melanocortin-4 receptors</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>5-MTHF</td>
<td>5-methyl-tetrahydrofolate</td>
</tr>
<tr>
<td>5,10-MTHF</td>
<td>5,10-methylenetetrahydrofolate</td>
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<tr>
<td>MTHFR</td>
<td>Methyltetrahydrofolate reductase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MTF</td>
<td>Methyltransferase</td>
</tr>
<tr>
<td>α-MSH</td>
<td>α-melanocyte stimulating hormone</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>Ngn3</td>
<td>Neurogenin-3</td>
</tr>
<tr>
<td>Nhh</td>
<td>Nescient helix-loop-helix</td>
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<tr>
<td>NPC</td>
<td>Neural progenitor cell</td>
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<td>NPY</td>
<td>Neuropeptide-Y</td>
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<tr>
<td>NSC</td>
<td>Neural stem cell</td>
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<tr>
<td>Ob-Rb</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal 5’-phosphate</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelancortin</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended daily allowance</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized control study</td>
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<tr>
<td>RF</td>
<td>Recommended folic acid</td>
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<tr>
<td>SAH</td>
<td>s-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>s-adenosylmethionine</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Six3</td>
<td>Rax in SIX homeobox3</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
</tr>
<tr>
<td>UL</td>
<td>Upper limit</td>
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<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
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*Selected for Oral Presentation at Experimental Biology Annual Meeting, April 22-26, Chicago, IL.

Manuscripts in preparation:

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Yang NV, Pannia E, Anderson GH. Too much of a good thing: A review of gestational folic acid on the neuronal development of homeostatic circuits.
CHAPTER 1

INTRODUCTION

Since the 1970s, there has been a rapid increase in the prevalence of obesity in North America [1]. The causes for this increase are recognized to be multifactorial; diet, exercise and lifestyle modifications have been at the forefront of approaches to preventing and treatment of obesity. In the past 20 years it has been recognized that maternal diets, through fetal programming, contribute to obesity and metabolic syndrome in the offspring. Most attention has been given to macronutrients on excess food intake and body weight gain during pregnancy [2]. Although less recognized as a causative factor, vitamin consumption during pregnancy has increased in the past 20-30 years, often exceeding daily intake recommendations [3-5], due to food fortification policies and availability of vitamin supplements. To reduce the risks of neural tube defects and embryonic delays, folic acid (the fully oxidized, synthetic form of folate, vitamin B9) has been added to refined wheat flour to increase intake in North America [6]. Combined with clinical advice to consume vitamin supplements with folic acid prior to and during pregnancy, results in many women in North America exceeding the tolerable upper intake levels (>1000μg/day) [3-5, 7].

Although there is little evidence on which to base concern that these high intakes are having adverse effects on human health, our studies have shown that when pregnant Wistar dams were fed a high 10-fold multivitamin diet, male offspring became obese and developed metabolic syndrome characteristics in later life [8]. Folic acid alone was identified to be primarily responsible for the phenotypic effects arising from the high multivitamin gestational diets. Methyl group vitamins including folic acid are co-factors in 1-carbon metabolism and the
metabolic phenotype of the offspring from high vitamin fed dams is associated with epigenetic modifications (DNA methylation) of genes involved in the hypothalamic food intake regulatory pathways [9]. These results support the potential link between excess folic acid intakes during pregnancy and the increased risk for obesity in offspring later in life.

The objective of this study was to determine the effect of folic acid content in gestational diets on the in utero development of hypothalamic food intake regulatory pathways measured at birth, and food intake and body weight of offspring at nine weeks of age.

This study explored three questions. First, does folic acid content of the gestational diet affect in utero development of neurons involved in food intake regulatory pathways? Second, does folic acid in the gestational diet affect the food intake and body weight of offspring up to nine weeks of age? Third, do the effects of gestational folic acid intakes on in utero development of food intake regulatory pathways to birth persist in adult offspring in later life?

We hypothesized that folic acid alters the in utero development of hypothalamic food intake pathways, and these effects persist, favouring increased food intake and higher bodyweight, in male adult offspring.
CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Childhood obesity in North America is classified as an epidemic, as obesity rates have continued to escalate over the years. Of the many determinants of obesity, changes in the maternal environment, including pre- and post-natal nutrition, has gained increased interest as nutrient status in utero can alter the long-term developmental trajectory of the offspring. While most researches focus on in utero programming effects of high macronutrient intakes, little is known about the effects and mechanism(s) of action from high micronutrient intakes commonly consumed during this period. The following literature review addresses this gap and highlights recent research evaluating the potential negative health effects associated with high doses of micronutrients during human pregnancy. The first sections of this review provide an overview of childhood obesity and micronutrient intakes during pregnancy. These sections are followed by a discussion on the programming effects of nutrients during pregnancy, with emphasis on the physiological role of the commonly consumed micronutrients, particularly folic acid (synthetic folate, vitamin B9) and other B-vitamins. A detailed review on the regulation of energy balance with focus on the central nervous system is presented and followed by a synopsis of the development of central hypothalamic food intake regulatory pathways. The final sections discuss the potential mechanisms by which folic acid consumption may impact the development of the brain, specifically the hypothalamus, of the offspring during gestation and into adulthood. Lastly, a summary and rationale for the research is provided.
2.2 CHILDHOOD OBESITY: A Global Epidemic

Classified as an epidemic, the prevalence of obesity has rapidly increased since the 1980s [10], with over 35% of men and women being affected worldwide; equating to 2.1 billion of the Earth’s population [11,12]. Developed countries have the highest prevalence and in 2014, over 50% of the Canadian adult population was classified as overweight or obese [13]. In conjunction with the rise in adult obesity, childhood obesity has also become a major concern. Prevalence of childhood obesity in developed countries has increased significantly in both boys and girls, from 8.1% and 8.4% in 1980, to 12.9% and 13.4% in 2013, respectively [11,14]. The most recent Canadian Community Health Study completed in 2015 showed that childhood overweight and obesity in Canada approximates to 25% [15]. Despite recent implementation of public health outreach programs, which encourage healthy eating and active lifestyle choices, obesity trends continue to escalate without any indication of plateauing [16-18].

The rise in obesity is mirrored by increases in the prevalence of the development of a cluster of metabolic disorders identified as the metabolic syndrome [19-21]; characterized by Type-2 Diabetes Mellitus, insulin resistance and cardiovascular complications [22-28]. The complexity of this disease and related morbidities has made it the largest global health challenge today, requiring continued research towards identifying the associated risk factors for preventative measures. Maternal nutrition and its role in programming obesity is the focus of this thesis.
2.3 MATERNAL NUTRITION AND OBESITY

2.3.1 Early Life Nutrition and Developmental Health Trajectory

In 1825, Jean Anthelme Brillat-Savarin, a French gastronomist wrote in *Physiologie du Goût*, “Dis-moi ce que tu manges, je te dirai ce que tu es.” [29] *What you eat determines what you are.* Although an over-simplified statement, a mother’s daily nutrient supply can determine their health and well-being and that of their child. Imbalances in maternal nutrition, such as excess or deficient intakes of macro- or micronutrients, are considered to be the most potent environmental risk factor affecting the developmental trajectory of the offspring [30]. Other risk factors include, but are not limited to; sociodemographics, maternal biophysical factors, obstetric complications and psychosocial factors [31].

In accordance with the Developmental Origins of Health and Disease paradigm, changes in the intrauterine environment at any critical time during fetal development determine the phenotypes and pathologies into adulthood [32-38]. Thus, environmental influences such as nutrient intakes before and during pregnancy can pose long lasting effects on the epigenotype and phenotype of the offspring [39-44]. Early studies and historical events such as the Dutch Winter famine during World War II (1944-1945) have been a prime example of this ‘early life programming’ phenomenon [45-49]. Mothers who suffered from malnourishment during this time gave birth to offspring with low birth weights and higher prevalence of cardiovascular disease, glucose intolerance, type-2 diabetes and other characteristics of the metabolic syndrome later in their life [2,51]. This became the basis for Barker’s “thrifty phenotype” hypothesis, proposing poor fetal nutrition stimulates metabolic adaptations that encourage increased food intake and weight gain in a “poor” nutrient setting [52]. These effects have also been confirmed by data derived from
animal studies. For example, providing a maternal macronutrient (i.e. protein) restricted diet, 50% of the control diet, to pregnant rodents during the second week of pregnancy led to offspring with growth retardation at birth and a phenotype reflecting characteristics of the metabolic syndrome, including obesity, hypertension and glucose intolerance [18,21,53]. Furthermore epidemiological studies have shown that not only maternal under-nutrition but maternal over-nutrition may also lead to the development of an obesogenic (i.e. obese) phenotype and metabolic syndrome in offspring [54-59]. Thus, the role of maternal nutrition in utero is critical and delicate as both under- and over- nutrition may have a long-term consequence on the offspring’s metabolism and risk for development of chronic metabolic diseases later in life [60,61].

2.3.2 Micronutrient Status during Pregnancy: Beyond the Upper Limit

Meeting adequate nutrient intakes during pregnancy is critical to support fetal growth and maternal health [62]. Vitamins, minerals, and trace elements often supplemented during pregnancy include: vitamins A, B (1, 2, 6, 9 and 12), C, D, E, calcium, magnesium, iron, copper, manganese, and zinc (calcium pantothenate, biotin) [63]. Of these micronutrients, folic acid (synthetic folate, vitamin B9) is the most frequently recommended because its deficiency leads to neural tube defect in the offspring [64-66]. Mandatory food fortification with folic acid has reduced its prevalence by approximately 50% [67]. However, food fortification policies and widespread use of supplements in developed countries have led to intakes of certain nutrients even beyond the tolerable upper limit (UL), defined as the maximum amount of nutrient that can be ingested without known adverse effects. The increased consumption of supplements has been attributed in part to a mistrust of the food supply in North America [68-74]. In Canada, data from the Canadian Community Health Survey 2.2 reported supplement use in 40% of adult Canadians,
with a greater proportion of women than men [75,76]. From the Alberta Pregnancy Outcome and Nutrition cohort, in 2012 over 90% of pregnant women were consuming a multivitamin supplement in addition to a single nutrient supplement [77]. The increased availability and accessibility of supplements [78] combined with food fortification [79] and dietary counseling that encourages vitamin consumption during pregnancy, has led to widespread and uncontrolled intakes of vitamins in women of child bearing age. In consequence, intakes of B-vitamins including folic acid, vitamin B12, and vitamin B6 in women within Canada and the USA have been reported to be 2.5-7 times greater than the recommended daily allowance (RDA; folic acid = 400-800µg/day) [7,80]. What was intended to be a strategy to correct micronutrient deficiencies could potentially have unintended and adverse health consequences.

2.3.3 B-Vitamin Consumption during Pregnancy

Public health professionals encourage women of childbearing age to consume a minimum of 400µg of synthetic folic acid daily, in addition to dietary folate found in food [82]. The Canadian Society of Obstetricians and Gynecologists in 2007 recommended women at risk of deficiency could safely consume 5,000µg/day, 10-fold higher than the RDA, of folic acid for optimal prevention of neural tube defects [83-85]. However, in 2015, a new report recommended women at moderate risk of neural tube defects to consume 1,000µg/day folic acid and only at high risk should pregnant women be consuming a maximum of 4,000µg/day folic acid [86]. Thus, folic acid intakes often surpass the tolerable upper intake level (>1000µg/day) [87], reflected in high blood folate levels. As a result, folate deficiency is virtually non-existent in the Canadian population. In 40% of the female participants of the 2007-2009 Canadian Health Measures Survey, high blood folate concentrations (>1360nmol/L) were reported and 78% of women at childbearing age had optimum risk-reduction of neural tube defects (>900nmol/L) [5]. However,
higher intakes seen in recent population trends currently pose unknown consequences on later child health.

2.4 B-VITAMINS IN THE ONE-CARBON PATHWAY

B-vitamins are critical during pregnancy for optimal fetal development through their essential roles in an interrelated pathway known as the 1-carbon cycle, seen in Figure 2-1. The 1-carbon pathway is involved in nucleotide synthesis, biological methylation reactions and maintenance of homocysteine level, all of which influence the development and health of the offspring [89]. Through these processes, the 1-carbon pathway functions to regulate the expression of genes, amino acid synthesis, redox regulation and metabolism [90-93]. Folates, vitamin B6 and B12 are known to be co-factors involved in 1-carbon metabolism during early life programming of the offspring [94-96]. In vitro studies show deficiency or excess of any of these B-vitamin co-factors in the 1-carbon metabolism alter development of mammalian embryos [90, 97-100]. Thus, regulation of the 1-carbon pathway is dependent on an adequate pool of folates and its cofactors [101-103].

Dietary folate polyglutamates, the natural folates found in food, are first hydrolyzed into monoglutamates in the intestinal lumen [104]. Mucosal cells that line the gastrointestinal tract absorbs folate monoglutamates via proton-coupled folate transporters, which are then converted into 5-methyltetrahydrofolate (5-MTHF). In contrast, folic acid, the completely oxidized and synthetic form used in supplements and food fortification, undergoes a two-step reduction process before converting into 5-MTHF. Folic acid is converted into dihydrofolate (DHF) followed by tetrahydrofolate (THF) via the rate limiting enzyme dihydrofolate reductase (DHFR)
Serine hydroxymethyltransferase (SHMT) as well as pyridoxal 5'-phosphate (PLP) derived from the vitamin B6 cofactor riboflavin, catalyzes the reversible transfer of serine from THF to create 5,10-methylenetetrahydrofolate (5,10-MTHF) and glycine [106,107]. 5,10-MTHF is irreversibly reduced by 5,10-methylenetetrahydrofolate reductase (MTHFR) to become 5-MTHF [108-110]. 5-MTHF is known as the biologically active form of folate that enters into the bloodstream and is transported to peripheral cells. 5-MTHF is also the only folate form that can cross the blood brain barrier [111-113]. During gestation, circulating 5-MTHF in the mother binds to placental folate receptors on the chorionic surface, which is then transported into the fetus [114,115]. As a result, peripheral and central organs take up 5-MTHF via reduced folate carrier (RFC) or folate receptor (FR). In peripheral cells, 5-MTHF is demethylated into THF by 5-MTHF-homocysteine methyltransferase (MTR) and vitamin B12-derived methylcobalamin as a coenzyme [104, 116].

During the demethylation of 5-MTHF into THF, the methyl group from 5-MTHF is transferred to homocysteine to convert into methionine, linking the folate cycle with the methionine cycle within the 1-carbon pathway [90,103,117,118]. Elevated levels of plasma homocysteine (>100 μM), termed hyperhomocysteinemia, is a key biomarker of folate as well as B6 and B12 deficiencies [110,119,120]. Homocysteine can also be methylated to methionine via a folate independent pathway which includes a methyl group from betaine (derived from choline) and is catalyzed by the enzyme betaine homocysteine methyltransferase (BHMT) [121,122]. Methionine is then adenosylated to s-adenosylmethionine (SAM), the universal methyl donor for the methylation of DNA, RNA, proteins, phospholipids, histones, neurotransmitters, neural stem cells (NSCs) and neural progenitor cells (NPCs) [123-125]. SAM is responsible for genome stability and gene transcription, localization of protein, and small molecule degradation [125-
After donating its methyl group for DNA methylation, SAM is demethylated into s-adenosylhomocysteine (SAH) and hydrolyzed back into homocysteine by S-adenosylhomocysteine hydrolase, where it is either metabolized by a B6-dependent process into amino acid cysteine, or re-methylated into methionine. The SAM:SAH ratio is an index of methylation and a low ratio due to low intakes of B vitamins may lead to hypomethylation of DNA and dysregulated gene expression [129-131]. On the contrary, excess intakes of these vitamins may increase the SAM:SAH ratio, resulting in hypermethylation of DNAs [132]. Thus, the 1-carbon metabolic pathway is essentially a tightly regulated flux of methyl intermediates and co-factors leading to early life epigenetic programming via the methylation of DNA and de novo synthesis of nucleotides [123,124,133,134].
Figure 2-1. Methyl vitamins, cofactors and enzymes involved in the one-carbon metabolic pathway.

Abbreviations: Vitamin B6 (B6); Vitamin B12 (B12); Vitamin B2 (B2; riboflavin); dihydrofolate (DHF); tetrahydrofolate (THF); hydroxymethyltransferase (SHMT); methylenetetrahydrofolate dehydrogenase (MTHFD); methylenetetrahydrofolate reductase (MTHFR); choline dehydrogenase (CHDH); betaine-homocysteine methyltransferase (BHMT); dimethylglycine (DMG); methylenetetrahydrofolate-homocysteine methyltransferase or methionine synthase (MTR); methionine adenosyltransferase (MAT); S-adenosylmethionine (SAM); S-adenosylhomocysteine (SAH); DNA methyltransferase (DNMT). Modified from [135].
2.4.1 Epigenetics: Emphasis on DNA Methylation

Epigenetics is defined as the process by which heritable changes in gene expression and phenotype are induced by environmental factors (i.e. diet) without affecting the underlying nucleotide sequence [136-138]. Through epigenetics, cell-specific gene expression responds to extracellular environmental signals such as gestational vitamins that can further influence downstream cellular pathways [139-142]. Epigenetic modifications encompass DNA methylation, covalent histone modifications, non-covalent mechanisms such as the addition of histone variants and nucleosome remodeling [143].

DNA methylation in particular is regulated through the 1-carbon pathway, which can modulate gene expression and is affected by the flux of nutrients available for 1-carbon transfer reactions. DNA methylation involves covalent modifications to cytosine residues on genomic DNA, known as palindromic cytosine-phosphate-guanine (CpG) dinucleotide sites located near promoter regions of genes, which may inhibit binding of regulatory proteins such as transcription factors, preventing gene activity [133,144]. Thus, alterations in availability of 1-carbon nutrients during critical windows of development, from gametogenesis, embryogenesis, and infancy and even through post-natal life can impact later-life development and phenotype [145-150].

2.4.2 Gestational B-Vitamins Promotes Fat Gain:

Support from animal and clinical studies have shown a significant link between intakes of B-vitamins involved in the 1-carbon cycle and the development of obesity and metabolic syndrome characteristics. (A summary of these animal and human studies are reported in Appendix 1.) In 2008, a large cohort study in India showed that mothers supplemented with folic acid had high erythrocyte folate concentrations. In addition, those with low serum vitamin B12 levels gave
birth to children who developed characteristics of the metabolic syndrome, including insulin resistance, at eight years of age [151].

In 2008, Szeto et al. first reported that high multivitamin gestational diets, 10-fold above recommended levels (high, non-toxic amount); fed during pregnancy to Wistar dams resulted in increased food intake, obesity, and characteristics of metabolic syndrome in mature offspring; males and females [92,152]. Methyl group vitamins were later found to be responsible for the phenotypic effects observed in the high multivitamin gestational diets, possibly through their role in DNA methylation of central feeding pathways in the brain [9]. When a 10-fold higher level of folic acid diet was fed to pregnant Wistar dams, male offspring post-weaning showed lower mRNA expression of Pomp (40%, p=0.03), an appetite-suppressing marker of food intake in the hypothalamus, compared to the control group fed recommended levels of folic acid. Epigenetic modification was supported by higher global methylation as well as Pomp-specific methylation in the hypothalamus of these male offspring. Therefore, lower Pomp mRNA expression and possible downstream effectors may have contributed to subsequent changes in the offspring’ phenotype [8]. This study was the first to show that epigenetic reprogramming of the food intake regulatory pathways occur in utero, altering function of the homeostatic food intake regulatory system in later life.
2.5 CENTRAL REGULATION OF ENERGY HOMEOSTASIS

Energy homeostasis is a tightly regulated process that balances energy intake and energy expenditure, by a set of complex and redundant neural networks and gene interactions [153-155]. This section provides an overview of the homeostatic regulation of energy balance in the brain and the key players involved, as depicted in Figure 2-2.

Figure 2-2. Simplified diagram of the food intake regulatory pathways in the hypothalamus

Abbreviations: arcuate nucleus of the hypothalamus (ARC); paraventricular nucleus (PVN); lateral hypothalamus (LHA); ventromedial hypothalamus (VMH); neuropeptide Y (NPY); agouti-related protein (AgRP); pro-opiomelanocortin (POMC); cocaine and amphetamine regulated transcript (CART); alpha-melanocyte stimulating hormone (α-MSH); leptin receptor (ObR); insulin receptor (InsR); Melanocortin receptor 4 (MC4R). Modified from [156].
2.5.1 Central Nervous System: Center of Energy Regulation

The feeling of hunger or satiety is dependent on signals from sensory properties of food, mechanical and chemical receptors in the gastrointestinal tract, as well as circulating metabolites and hormones that integrate into the central nervous system to tell your body to stop or start eating [153,155,157]. Regulation of food intake and energy homeostasis involves many peripheral and central circuits, including: the gastrointestinal tract, pancreas, liver, muscle, adipose tissue, and the brain. However, it is in the hypothalamus, a central regulatory network in the brain that plays a critical role in communicating nutrient signals to other neural areas and the periphery. The hypothalamus consists of many neurocircuits that decipher nutritional and hormonal signals from the environment, translating them into physiological or behavioural responses that favour energy balance; a process which ensures food intake regulation is precise and robust [158].

Lesions in the hypothalamus, specifically the ventromedial and arcuate nucleus (ARC), have led to cases of obesity [159], including a few studies on the lateral hypothalamus that have indicated weight loss; supporting the role of the hypothalamus as a primary regulatory of food intake. Termed as the “major hub”, the ARC consists of many feeding-related neuronal networks and pathways, analyzing input signals from peripheral organs and mediating output signals to ensure a balanced energy homeostasis [153]. The majority of the food intake neuronal markers are concentrated within the ARC, projecting out to the paraventricular, dorsomedial nuclei, and the lateral hypothalamic area, to stabilize feeding and energy expenditure [160-162]. It is critical that the ARC as well as the other hypothalamic areas be fully developed in order to ensure energy balance throughout life.
2.5.2 Food Intake Regulatory Neuropeptides

Within the arcuate nucleus are two populations of neurons that undergo antagonistic innervation to ensure a balanced energy homeostasis [163-166]. Orexigenic peptide-containing neurons, neuropeptide Y (NPY) and agouti-related peptide (AgRP) are known to stimulate food intake and weight gain when activated [163,167]. NPY and AgRP activity increase during fasting, prior to feeding, and gradually decline with food intake [168]. On the contrary, anorexigenic peptides, consisting of pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) containing neurons, inhibit food intake and promotes weight loss [157-156,161,169-171]. Their location within the ARC makes them primary players in maintaining energy balance through the orchestration of adaptive behavioural, autonomic, and endocrine responses [153,158]. As a result, peripheral information is sent to each of these neuropeptide-containing neuron populations to either activate or inhibit downstream effectors of the food intake regulatory pathways.

Both POMC and NPY neurons have been studied extensively as the central regulatory neurons of energy balance [172-174]. The POMC polypeptide precursor is derived from the melanocortin, α-melanocyte stimulating hormone (α-MSH). A-MSH binds to and activates neuronal melanocortin-4 receptors (Mc4R) on axon terminals of neurons, decreasing food intake and favouring weight loss in response to abundant energy intake [175-178]. The absence of α-MSH or Mc4R in POMC knockout mice models has resulted in hyperphagia and obesity as well as insulin resistance [179-180]. Due to POMC neurons being the most potent neuropeptides, slight changes in the POMC neuronal population can significantly impact metabolism and alter central regulation of food intake [181]. The second group of neurons that regulate energy balance consists of NPY neurons that are co-expressed with AgRP neurons. Deletion of NPY and AgRP
genes does not affect food intake regulation comparable to POMC, however mild alterations in energy balance are evident [182,183]. Pierce and Xu identified compensatory mechanisms developed during the loss of AgRP neuropeptides in mice, via increased de novo cell proliferation of hypothalamic AgRP-containing neurons [184].

A tight relationship has been established between NPY neuropeptide secretion and hyperphagia. As the most dominant orexigenic neuropeptide in the hypothalamus, activation of NPY neurons increases motivation to feed, and reduces energy expenditure via the sympathetic nervous system [185]. Injecting NPY directly into the third ventricle of the hypothalamus, stimulated food intake, decreased energy expenditure and increased formation of white adipose tissue [186]. Opposingly, knockout of the Npy gene reduces hyperphagia and obesity [161]. Activation of NPY neurons also inhibits POMC neurons directly through NPY1- and γ-aminobutyric acid-A (GABA)-receptors. Failure in this reciprocal inhibitory system between NPY and POMC neurons leads to a deregulated feeding pathway [188-189]. The relationship between these two neuronal populations is also projected widely to second- and higher-order neurons located in the brain and spinal cord [153,191]. Secondary interactions are found in common downstream target sites in the paraventricular nucleus, such as the melanocortin-4 and melanocortin-3 receptors. Disruption of melanocortin signaling to downstream target neurons causes obesity [161,189-190].

Although the reports are few, development and balance of these two neuronal populations is affected by maternal nutrition and reflected in the phenotype of the rat offspring. For example, maternal high-fat diets fed to pregnant mothers resulted in neurodegeneration of POMC-containing neurons as well as permanently exacerbated orexigenic pathways [238,239] in the hypothalamus of their offspring, which were hyperphagic and obese [219,240,241]. Food restriction of pregnant rat dams during gestation also results in offspring with hyperphagia and
reconfigured food intake regulatory pathways, specifically reduced POMC and increased NPY expression in the arcuate nucleus [245,246]. Food restriction during pregnancy by 50% of ad libitum resulted in restricted brain growth due to decreased neurogenesis and stunted migration of neurons to the hypothalamus, during the late embryonic period in rats [53,247-249].

2.6 DEVELOPMENTAL TRAJECTORY OF THE FOOD INTAKE REGULATORY PATHWAY

The early developmental stages of life, from prenatal to early postnatal days, represent a critical developmental window that determines the regulation of food intake and energy homeostasis of the offspring for the rest of their life [158]. During this fetal to neonatal time period, brain growth and development is rapid [2] and vulnerable to maternal diet [192].

2.6.1 Animal Models

Animal models have been a valuable tool to understanding the mechanisms of developmental programming and trajectory that occurs in early life, allowing investigators to control conditions of the fetus at various stages of development [2]. Through animal studies, it has been shown that the timing and sequence of in utero early life development in the brain is highly conserved among mammalian species, as variability in neurodevelopment increases with age after birth [193-194]. Rodents are the most preferred models for studying neurodevelopment, specifically in the hypothalamus, as both humans and rodents share periods and sequence of brain maturation, neuronal differentiation and active adipogenesis in common. In addition, the advantage of using rodent models includes a short gestation period and lifespan, cost effectiveness, and control over
genetic and environmental variability. As a result, rodents are the predominant species of choice in identifying the relationship between maternal nutrition and developmental programming [2].

Although the similarities are many between rodent and human in utero neurodevelopment, differences begin to appear in the last week or trimester of gestation, where human development is accelerated and hypothalamic feeding circuits are extensively developed in utero. In contrast, newborn rodents are therefore more immature than humans at birth. Development of the hypothalamus continues into postnatal stages of life, up to weaning, when pups begin to consume solid food and neurons in the hypothalamus begin to innervate from the arcuate nucleus to downstream hypothalamic targets [195-199]. Projections from the ARC to dorsomedial hypothalamic region was the first to develop by post-natal day 6, followed by the neurons in the paraventricular hypothalamus, from P8-P10, and lastly the innervations to the lateral hypothalamic area occurred at P12 [195,196].

2.6.2 Neural Stem Cells and Neural Progenitor Cells

The development of neurons involved in the food intake regulatory pathways during early life, originate in the hypothalamus. The hypothalamus consists of a highly neurogenic region and a progenitor pool that can generate neural stem cells (NSCs) and neural progenitor cells (NPCs) into neurons important for the regulation of energy balance, including NPY, AgRP, POMC, and CART-expressing neurons [184,200,201]. The structural and functional development of neurons that regulate the hypothalamic feeding circuitry is determined by intrinsic transcription or growth factors as well as morphogens [202-204]. Transcription factors, identified in stem cell cultures, have been shown to regulate neuronal development through early critical processes of neurogenesis, proliferation, differentiation, and looping [30].
The early developmental stages of life originate from stem cells, which are then guided by transcription factors. Development of the hypothalamus initiates in the proliferative zone of the third ventricle, containing the neural progenitor pool for all neurons involved in the food intake regulatory pathways [205]. Neural stem cells (NSCs) are highly proliferative cells that have the ability to regenerate themselves into new NSCs or differentiate into neural progenitor cells (NPCs) that later become mature neurons, glial cells, or astrocytes. Animal studies have shown that the behavior of NSCs can be influenced by environmental factors such as exercise and stress during pregnancy; affecting proliferation, survival and neurogenesis of these NSCs [206]. Transplanting exogenous NSCs as a means of replacement therapy can lead to the suppression of neurodegenerative diseases [207-209]; as well as mitigate feeding dysfunction due to neurodegeneration in the hypothalamus, such is the case in obesity [210,211] and Huntington’s disease [212,213].

After NSCs differentiate into and give rise to embryonic NPCs in the ventricular zone, they migrate to the third ventricle forming the hypothalamus [205]. The origin of hypothalamic neurons has been a controversial topic as two theories have been considered: 1) they may either originate from resident NPCs in the hypothalamus or, 2) originate from non-resident cells that migrate elsewhere to the hypothalamus during neurodevelopment [214,215]. By the time they become NPCs in rat embryos, they are already expressing feeding-related neuropeptides. These NPCs located within the hypothalamus undergo further division, migration, proliferation, and differentiation into mature neuro-endocrine neurons, from E (embryonic day) 12-16. These processes are driven by multiple intrinsic and extrinsic cell signals, including proneural transcription factors, environmental factors, as well as the orexigenic stimulus ghrelin, and the anorexigenic stimulus leptin [202,216-218]. Transcription factors promote proliferation of these
NPCs, expanding the NPY and POMC neuron populations, as well as supporting differentiation into mature neurons [219].

2.6.3 Cell Fate of POMC and NPY Neurons

During early life development, neurons differentiate and mature following a fixed schedule based on their cell lineage. The majority of hypothalamic NPCs express NPY, AgRP, POMC, and CART by E18 in the rat embryo (Figure 2-3) [220]. POMC-expressing neurons were the first to be detected in the rat hypothalamus at E10.5 [221]. However, the majority of immature POMC-containing neurons adopt a non-POMC fate, as NPY-expressing neurons became evident and the number of POMC neurons decreased drastically [222]. This suggests that nearly one quarter of NPY neurons share a common progenitor with POMC neurons, derived from the POMC-expressing lineage. In turn, POMC precursor cells begin to assume a NPY/AgRP phenotype by E13.5-E14.5 [223-225]. The gradual decrease in POMC neurons and increasing onset of NPY neurons represents a highly plastic maturation process that begins at E10.5 up to early postnatal life by P (postnatal day) 15, when rodents have fully matured. [223,226]. As several neuronal cell types arise from the POMC-expressing lineage during early life development, this suggests that environmental factors could influence cell fate decisions during this critical window in gestation. These factors could permanently affect the neuronal composition and makeup of feeding-related circuits in the hypothalamus.

The complexity in developing a balanced energy and food intake system is dependent on various transcription factors and neurotropic hormones involved in directing cell fate to maintain homeostasis. Transcription factors, including neurogenin-3 (Ngn3), rax in SIX homeobox3 (Six3), nescient helix-loop-helix (Nhh) and Mash1, are crucial to development to maturity of feeding-
related neurons [202,224,227-229]. In addition to transcription factors, neurotrophic hormones such as leptin modulate the development of NPY and POMC neurons [53,230]. From as early as E10.5 in mice [231] and E14 in rats [232], leptin receptors, *Ob-Rb*, are present in the embryonic brain [53]. Genetically modified leptin-deficient (ob/ob) mice have shown decreased synaptic density and reduced firing of action potentials in NPY/AgRP and POMC/CART neurons of hypothalamic neural circuits [196]. In addition to transcription factors and neurotropic hormones, recent studies have confirmed neuronal expressions in the ARC region are sensitive to external influences such as the gestational diet during pregnancy [223]. Therefore the gestational diet must be studied in order to investigate its possible influences on the proliferation and differentiation of ARC neurons.
Figure 2-3. Critical windows of development of food intake regulatory neurons in rats.

Abbreviations: embryonic day (E); neuropeptide Y (NPY); agouti-related protein (AgRP); pro-opiomelanocortin (POMC); cocaine and amphetamine regulated transcript (CART); leptin receptor (ObR); insulin receptor (InsR).
2.7 FOLATE AND NEURONAL DEVELOPMENT: CURRENT EVIDENCE

The generation of neurons through neuronal development occurs over a wide-range of processes encompassing: neurogenesis, synaptogenesis, and early myelination of the fetal brain from the third and last week of embryogenesis to early post-natal life. Late-pregnancy perturbations via gestational diet and nutrient delivery through the placenta during these periods of high fetal demands will strongly impact neuronal development of the hypothalamus [16,53,247-249]. Current evidence indicates that gestational folic acid affects initial stages of NSC proliferation and differentiation, but nothing has been studied beyond these early developmental stages. Therefore, by examining later stages of neuronal development, from birth to early post-natal life, can we then determine the full scope of influence gestational diet has on development of hypothalamic food intake pathways.

Many studies have explored the effects of maternal dietary folic acid intakes on post-natal development of the hippocampus [270], septum, and cortex, as well as the ventricular and subventricular zones [271], leading to apoptotic cell death and neurobehavioural consequences later in life [89,255,272,273]; but not hypothalamic pathways affecting food intake and metabolism. Folic acid is vital to neurodevelopment, as both high and low concentrations exposed in utero can affect intracellular signaling pathways involved in the development of the central nervous system from gestation to later life.

Folic acid is known to affect NSC proliferation and differentiation in brain cortices, during development of the central nervous system [91,251-254]. When comparing a recommended maternal diet (2mg folic acid/kg diet) to a folic acid-deficient diet (0mg folic acid/kg diet), from E11-E17, a significant decrease in NSCs proliferation and increase in apoptosis by E17 was
found in the brain of mice [255]. In rhesus monkey embryonic stem cells, decreased cell proliferation and neuronal differentiation also occurred when mothers were fed diets depleted in folic acid [256]. In cultures of embryonic NSCs from rat fetuses, maternal folic acid deficiency led to a reduction in cell proliferation impairing mitosis and increasing apoptosis in neuronal cells [218,257]. Folic acid supplementation stimulated these cells to proliferate [251].

*In vitro* studies using neurosphere cultures of NSCs derived from rodent embryos have also linked gestational folic acid consumption to altered transcription factors in intracellular signaling pathways that modulate proliferation and differentiation of NSC pools into newly generated neurons in the brain during gestation [218,253,258]. Liu and others confirmed and highlighted folic acid to induce a dose-dependent response on the *Notch* signaling transcription factor that promotes proliferation and differentiation of embryonic NSCs [251,259,260]. Maternal folic acid deficiency decreased NSCs differentiation and increased apoptosis of neurons in the fetal brain via the *ERK1/2* transcription factors involved in the *MEK1/2* signaling pathway [252,257,261,262]. *ERK1/2* genes are expressed in all neurons within the brain and are associated with cell growth, proliferation, differentiation and migration, as well as possibly regulating synaptic remodeling, axonal growth, long term potential formation and neuronal excitability [218,251,263-265]. Studies of various cell types indicate that the deleterious effects of folate deficiency may result from lowered levels of purines, thymidine, and methionine, raised levels of homocysteine, or changes in intracellular signaling pathways [266-269]. However, the effects of folic acid on the development of hypothalamic food intake pathways in particular have not been reported yet; the focus of the present research.
2.8 SUMMARY AND RATIONALE

Understanding the effect of high intakes of folic acid on the brain and its regulatory pathways is needed to provide evidence to support evaluation of dietary advice and prenatal supplements for women. However, how folic acid affects and regulates \textit{in utero} development of the hypothalamic food intake regulatory pathways has received little investigation. In the rat model, it is clear that high intakes of folic acid during gestation affect development and function of these pathways and lead to characteristics of metabolic syndrome in the offspring, male and female. Therefore, the goal of this research is to add to an understanding of how gestational folic acid, at both low and high intakes, affects development of NPY and POMC neurons to birth and their contribution to characteristics of metabolic syndrome in the offspring. By understanding how gestational folic acid content affects the development of the food intake regulatory pathways from gestation to adulthood, using the Wistar rat model, may we then potentially create new opportunities for therapeutic interventions to reduce the risk of childhood obesity and metabolic syndrome characteristics.
CHAPTER 3

HYPOTHESES AND OBJECTIVES

3.1 Overall Hypothesis

Folic acid content in gestational diets affects *in utero* development and function of hypothalamic food intake pathways, favouring an obesogenic phenotype in the male offspring.

3.2 Overall Objective

To determine the effects of folic acid content in gestational diets on hypothalamic food intake regulatory pathways of the male offspring at birth and post-weaning.

3.3 Specific Hypotheses and Objectives

**Specific Hypothesis**

(1): Folic acid intake, below and above requirements, in pregnant Wistar rat dams affects *in utero* development of NPY and POMC neuron populations in male offspring.

(2): Gestational folic acid intakes above requirements lead to increased food intake and weight gain of male offspring post-weaning.

**Specific Objectives:** To compare the effects of gestational diets containing folic acid provided at 0, 1.0 (control), 2.5, 5.0, or 10-fold of recommended levels on: (1) birth weight, hypothalamic *Npy* and *Pomc* gene expression, neuron maturation and activation at birth, and (2) post-weaning body weight and food intake, and neuron expression and function of NPY and POMC in the arcuate at 9 weeks of age.
4.1 Abstract

**Background:** Folic acid plays an important role in early brain development of offspring, notably on neural stem cell proliferation and differentiation. Excess (10-fold) intakes of folic acid in the gestational diet have been linked to increased food intake and obesity in adult rat offspring.

**Objectives:** To determine the effects of folic acid content in gestational diets on the development and function of hypothalamic food intake regulatory pathways of the male Wistar rat offspring at birth and post-weaning.

**Methods and Design:** Pregnant Wistar rats were assigned to AIN-93G diets (n=12-14/group) containing folic acid at: 0, 1.0 (control), 2.5, 5.0, or 10.0-fold of the recommended level. Expression of NPY and POMC neuronal populations were assessed in coronal hypothalamic sections from male rat offspring collected at birth and 9-weeks post-weaning.

**Results:** A dose-related effect ($P<0.01$) was identified between gestational diet folic acid content and expression of mature NPY-, but not POMC-containing neurons in the hypothalamus, at birth. Pups from the 5.0-fold folic acid group had 3.6-fold more mature NPY-neurons than control. However, gestational folic acid content had no effect on the number and maturation of POMC-expressing neurons. Birth weight was higher in all treatment groups compared to control ($P<0.01$). Differences in body weight and food intake progressed into post-weaning ($P<0.001$). At 9-weeks post-weaning, increased body weight and food intake in 2.5-, 5.0-, and 10.0-fold
folic acid groups were negatively associated with activation of POMC-expressing neurons in the arcuate nucleus in their respective groups (P<0.05).

**Conclusion:** The development of hypothalamic NPY neurons *in utero* and POMC neurons at maturity reflects the folic acid content in gestational diets, which were consistent with higher food intake and body weight in the male offspring.

### 4.2 Introduction

Childhood obesity has become a global health challenge. It is becoming more ubiquitous as one-in-five children in Canada and the US are overweight or obese [15,280]. Globally, the number of overweight children under the age of five was estimated to be over 42 million in 2015 [335]. According to the Developmental Origins of Health and Disease paradigm, the maternal environment during pregnancy programs the offspring’s development and later life phenotype including obesity and characteristics of the metabolic syndrome [22,39-43,281,282]. However, it is unclear if this occurs because of: environmental variables after birth, central neuronal development of systems regulating behavior (e.g. food intake) and metabolism (e.g. glucose, hormones) in the offspring [202,283,284] alone, or their interaction.

The hypothalamus plays a critical role in regulating food intake and maintaining energy homeostasis. Situated in the hypothalamus, the arcuate nucleus (ARC) contains two key food intake regulating neuronal populations; the orexigenic neuropeptide Y (NPY) neurons and the anorexigenic pro-opiomelanocortin (POMC) neurons [285,286]. During gestation, the hypothalamus is highly sensitive to the *in utero* environment as neural cells differentiate and proliferate into a complex neuronal network controlling energy balance [30]. Many nutrients in
the maternal diet have been shown to induce epigenetic alterations of genes in the brain [218,223,161,287-289].

Folic acid (synthetic folate, vitamin B9) is a methyl metabolism co-factor that plays an essential role in early development of offspring, regulating gene expression and rapid cell growth and proliferation via epigenetic (i.e. DNA methylation) reprogramming [250,290,291]. Folate deficiency during pregnancy leads to neural tube defects and embryonic delay in the offspring [292], however excess intakes during pregnancy also affect the long-term phenotype. Altered development of genes expressing feeding-related neuropeptides by folic acid has been reported by several animal studies to date [161,287-289]. We have previously shown that feeding high multivitamins (HV, 10-fold of recommended multivitamin level) and high (10-fold) folic acid to pregnant Wistar rats, resulted in male and female offspring with increased food intake, obesity and characteristics of the metabolic syndrome post-weaning [92,152]. These diets also altered DNA methylation patterns in hypothalamic feeding neuropeptides post-weaning, associated with the higher food intake and body weight. Together these studies highlight the in utero programming effects of excess (10-fold) vitamins, specifically folic acid, towards obesity in the offspring. However, the impact of folic acid during gestation on cell development in feeding pathways at birth and the relationship between these outcomes and regulation of food intake at maturity is unknown.

Therefore, we hypothesized that folic acid content in the gestational diet alters development of food intake pathways in utero to favor obesity in the offspring. Our objectives were twofold: first to determine the effects of gestational folic acid below and above requirements on the
development of the hypothalamic feeding-related neurons, NPY and POMC, in rat offspring at birth. Second, to determine the relationship between the development of these food intake regulatory pathways at birth, to their regulation of food intake and phenotype at 9-weeks post-weaning. Food intake, body weight, hypothalamic folate levels, NPY and POMC gene and protein expression and activity were measured.

4.3 Materials and Methods

Animals and Diets

First-time, 2- to 3-days pregnant Wistar rats were purchased from Charles River Farms (Quebec, Canada) and were housed in individual cages upon arrival. Animals were placed in a temperature controlled room, at 22 ± 1°C and maintained on a reverse 12h dark-light cycle (lights on at 0700hrs). Food and water were provided ad libitum throughout the entire study period. All experimental procedures were approved by the University of Toronto Animal Care Committee. During pregnancy until term, dams (n = 12-14/group) were randomly assigned to an AIN-93G diet [293] (energy density 3.8kcal/g) containing folic acid at: 1) recommended level (RF, 2mg/kg diet, control) [294] 2) 0-fold (low-0RF, 0mg/kg diet); 3) 2.5-fold (moderate-2.5RF, 5mg/kg diet); 4) 5.0-fold (high-5RF, 10mg/kg diet), or 5) 10.0-fold (very high-10RF, 20mg/kg diet) levels. All diets were purchased from Research Diets Inc. (New Jersey, USA) Diet compositions are shown in Table 4-1. At birth, 2-4hrs post-delivery, litters were culled to 6 unsexed pups per dam to minimize differences in milk availability. During lactation, all dams were placed on the control diet until weaning (21 days after delivery). At weaning, one male offspring from each litter was
randomly chosen, housed singly and placed on the RF diet for 9-weeks post-weaning. Figure 4-1 depicts the design of the study.

Table 4-1. Composition of experimental diets based on the AIN-93G diet.

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<thead>
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<th>Ingredient</th>
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<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>
| **Diet abbreviations:** AIN93G with: RF(recommended folic acid) diet = 2mg/kg folic acid; 0RF = 0mg/kg (no added) folic acid, 2.5RF = 5mg/kg folic acid, 5RF = 10mg/kg folic acid, 10RF = 20mg/kg folic acid.

Table 4-1. Composition of experimental diets based on the AIN-93G diet.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>0RF</th>
<th>RF</th>
<th>2.5RF</th>
<th>5RF</th>
<th>10RF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>g/kg diet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Fat</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td><strong>Ingredient</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein (&lt;85% protein)</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cornstarch</td>
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<td>397.486</td>
<td>397.486</td>
<td>397.486</td>
<td>397.486</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>132</td>
<td>132</td>
<td>132</td>
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<td>132</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Fiber (cellulose)</td>
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<td>70</td>
<td>70</td>
<td>70</td>
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<td>70</td>
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<td>t-Butylhydroquinone</td>
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<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td>Mineral Mix (S10022G)</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin Mix (V10037)</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Vitamin Mix (V14905), No Added Folate</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>0</td>
<td>0</td>
<td>0.003</td>
<td>0.008</td>
<td>0.018</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td><strong>Added Folic Acid (mg/kg)</strong></td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 4-1. Schematic sketch of the study (A) design and (B) protocol for male offspring from birth to 9 weeks post-weaning. Diet abbreviations: AIN93G with: RF(recommended folic acid) diet = 2mg/kg folic acid; 0RF = 0mg/kg (no added) folic acid, 2.5RF = 5mg/kg folic acid, 5RF = 10mg/kg folic acid, 10RF = 20mg/kg folic acid. Gestational and pup diets denoted at the top of figure. PW: Post-weaning. ↑ = weekly body weight & food intake.

**Body Weight and Food Intake**

Birth weight (g) of male offspring was recorded 2-4 hours post-birth. From weaning (week 0) to 9-weeks post-weaning, body weight and food intake (g/day) was measured weekly.
**Tissue Collection**

At birth and at 9-weeks post-weaning, male pups were sacrificed by decapitation 2-4hrs post-delivery or following a 6-hr day fast, respectively. Whole brains were rapidly removed and snap frozen on dry ice and stored at -80°C.

The hypothalamus was macro-dissected free-hand on ice as previously reported [295]. Using a sterile single-edge razor blade, a cut was made at the anterior portion of the hypothalamus using the optic chiasm as a reference and as a horizontal limit. The posterior hypothalamus and the mammillary bodies were considered a caudal section. The preoptic, supraoptic, paraventricular, anterior, suprachiasmatic, dorsomedial, ventromedial, arcuate, mammillary, posterior and lateral complex nuclei of the hypothalamus were contained within each block cut.

**Sex Genotyping**

Tail snips were retrieved from all Wistar pups sacrificed at birth and genotyped according to the method described by Clapcote & Roder [296]. Genotyping at the *Jarid1c* (X-chromosome-specific gene) and *Jarid1d* (Y-chromosome-specific gene) locus was performed to determine sex of pups, using the following *Jarid* primer set: Forward: 5’ – CTGAAGCTTTTGCTTTTGAG – 3’ and Reverse: 5’ – CCGCTGCAAAATTCTTTGG – 3’, purchased from ACGT Corp (Ontario, Canada). DNA from tail clips were extracted using MyTaq™ Extract-PCR Kit (Bioline, London, UK). PCR was performed under the following cycling conditions: 95°C for 3 minutes, followed by 35 cycles of 95°C for 15 sec, 60°C for 15 sec, and 72°C for 20 sec (GeneAmp®PCR Sysytem 9700, Ellange, Luxembourg). The PCR products were run on a 3.5% (w/v) agarose gel, for 1hr
and 45min at 135V. Male pups yielded two DNA fragments simultaneously of 331 base pairs (bp) and 302bp, while female pups exhibited a single fragment of 331bp.

**5-Methyltetrahydrofolate in Hypothalamus**

High performance liquid chromatography (HPLC) was performed to determine the concentration of 5-Methyltetrahydrofolate (5-MTHF) in the hypothalamus of Wistar rat male offspring at birth and post-weaning as described by Selhub and others [297-299]. Selhub compared HPLC to *Lactobacillus Casei* Microbiological Assay, a gold standard for folate analysis, confirming consistent results between the two methods. In addition, due to a loss of folate activity during incubation with chicken conjugase in the microbiological assay protocol, HPLC has a lower detection limit than microbiological assay, a difference of ~30% [300,301].

Hypothalamic brain sections were thawed on ice and immediately suspended in a cold extraction buffer of artificial cerebrospinal fluid (100μL/sample, Harvard, USA) with added stabilizer (50mmol/L potassium tetraborate, 10g/L sodium ascorbate, pH 9.2). Samples were then sonicated on ice and placed in a boiling water bath for 10min. The high pH of the extraction buffer prevented endogenous folate conjugase activity from occurring and the sodium ascorbate inhibited oxidative degradation of the folates. The extract was cooled in an ice bath and neutralized with 1mol/L monobasic potassium phosphate. The sonicated samples were centrifuged at 10,000rpm for 10min at room temperature. The supernatant was collected and immediately stored at -80°C until further use.

Samples were analyzed using the BAS 460 Microbore-HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN, USA) with a Uniget C-18 reverse phase microbore column as the stationary phase (Basi, Cat no. 8949). The mobile phase consisted
of 40mM sodium phosphate dibasic, heptahydrate, and 8% acetonitrile (v/v), acid, adjusted to pH 5.5 with 85% phosphoric acid (Sigma-Aldrich, Oakville, Canada), and was filtered through a 0.22μm pore membrane filter. Mobile phases and their reservoirs were changed daily. Mobile phase was delivered at a flow rate set at 0.9mL/min, adjusting potential between working electrode (Uniget 3mm glassy carbon, BAS P/N MF-1003) and Ag/Ag/Cl reference electrode to 550mV. Detection gain was 1.0μA, filter was 0.2Hz and detection limit was set at +50μA. For each sample, 5μL was injected into the HPLC system for analysis with the internal standard, theobromine (2ng/μL, Sigma Chemicals) as previously described in Giorgi et al.(2012). 5-MTHF (Merck Eprova AG, Schaffhausen, Switzerland) was used as an external standard to quantify and measure the peaks on the chromatograph. Retention time for 5-MTHF was ~ 7.1min and detection limit of 5-MTHF was determined by injection of 1mg/mL of pure 5-MTHF standard and diluted to known concentrations in the HPLC. Sensitivity was selected at the concentration where signal to noise ratio was 3:1.

**In Situ Hybridization**

Frozen whole brain samples were mounted on a stage with Tissue-Tek ® Optimum Cutting Temperature (OCT) Compound (Sakura ® Finetek USA Inc., California, USA). Using the Leica CM 3050S cryostat, brain samples were sliced and 20μm sections containing the hypothalamus from bregma A 2.9-2.3mm were obtained on Superfrost Plus Microscope Slides (Fisher Scientific, California, USA) [302]. Frozen sections were stored in air-tight slide boxes (Fisher Scientific, California, USA) in dry conditions at -80°C.

The protocol for *in situ* hybridization staining was a modification of a protocol previously reported [303]. Briefly, antisense *Pomc* and *Npy* digoxigenin-labeled riboprobes were generated
from plasmids containing PCR fragments of *Npy* and *Pomc* using the following oligonucleotide primers sets [304]: *Npy*: (Forward) 5’ – CTCCGCTCTGCGACACTAC – 3’, (Reverse) 5’ – AATCAGTGTCTCAGGGCT – 3’, *Pomc*: (Forward) 5’ – ACCTCACCACGGAGAGCA – 3’, (Reverse) 5’ – GCGAGAGAGTCGAGTTTGCA – 3’. Plasmids were generated from extracted DNA in hypothalamic brain samples of Wistar rats using Dneasy Blood & Tissue Kit (Qiagen, Hilden, Germany). DNA extracts were subject to RNA transcription using KAPA2G Fast HotStart ReadyMix PCR Kit (KAPA Biosystems, Massachusetts, USA), according to manufacturer protocol. The digoxigenin-labeled *Npy* and *Pomc* probes were generated by incorporating digoxigenin-labeled UTP from the DIG DNA Labeling Mix (Sigma-Aldrich, Missouri, USA).

Frozen sections were retrieved and thawed at room temperature. ImmEdge hydrophobic barrier pen (Fisher Scientific, Ontario, Canada) was used to seal the edges of the glass slides in order to retain solutions. To retrieve the antigen, brain slices were quickly digested with 20μg/mL proteinase K in pre-warmed 50mM Tris buffer solution for 1min. Slides were rinsed in Tris-buffer to remove proteinase K and were then immersed in ice-cold 20% (v/v) acetic acid for 20sec, in order for the probe and antibody to permeate the cells. Slides were then gradually dehydrated in ethanol, from 70% to 100% ethanol, followed by air drying. The digoxigenin probes were diluted to 1-2μg/mL hybridization solution containing: formamide, salt solution (NaCl, EDTA, Tris-HCl, sodium monobasic, sodium dibasic), Denhardt’s solution, dextran sulphate, heparin, and sodium dodecyl sulfate (SDS). The hybridization cocktail was applied to each slide and brain sections were incubated overnight (12-16hrs) in a humidified hybridization chamber at 65°C. During this step, the RNA probe hybridized to corresponding mRNA targets. The slides were then washed in formamide and saline-sodium citrate (SSC) buffer at 45°C for
5min each wash (total of 3 washes), to remove excess probe and hybridization buffer as well as non-specific and/or repetitive RNA hybridization. Sections were next incubated in goat polyclonal anti-digoxigenin primary antibody (1:100, No.ab76907; Abcam, Cambridge, UK) conjugated to 1% bovine serum albumin in Tris-buffer for 1hr at room temperature. Slides were washed in Tris-buffer three times. Secondary goat anti-mouse IgG (H+L) antibody, Alexa Fluor 555 (A-21422, Thermo Fisher Scientific, Massachusetts, USA) diluted in 1% bovine serum albumin-Tris buffer was added to the slides and incubated for 1hr. Slides were then rinsed with Tris-buffer and mounted with Lab Vision™ Permafluor™ Aqueous Mounting Medium (Thermo Fisher Scientific, Massachusetts, USA) and coverslips were applied. All procedures were done in an RNA-free environment.

**Immunohistochemistry**

To identify neuronal populations susceptible to folic acid intakes during pregnancy, we measured the expression of NPY and POMC containing neurons, known to increase or decrease feeding, respectively. In addition, we looked at the maturation of these neurons using the neuronal marker NeuN [181,305]. Food intake markers were also co-labeled with c-Fos, an immediate early gene marker to detect recent activity of the neuronal populations of interest [306]. Frozen whole brain samples were immediately placed into 4% paraformaldehyde (PFA; pH 7.4) and fixed overnight for 12hrs. The fixed brains were then transferred into a series of sucrose solutions from 10%, 20%, to 30%, with 2hr incubation between each transfer. Within a week, the Wistar brains were sliced into 20μm-thick serial coronal sections using a freezing microtome (Leica CM 3050S). All coronal sections containing the ARC from bregma A 2.9-2.3mm and using the fornix and third ventricle as landmarks [244,307], were mounted onto Superfrost Plus
Microscope Slides (Fisher Scientific, Ontario, Canada) and stored at -20°C.

Frozen sections containing the hypothalamus were thawed at room temperature and ImmEdge hydrophobic barrier pen was applied to the edges of the slides and left to dry for 15min. Sections were first permeabilized with 0.3% phosphate-buffered saline (PBS) – Triton X-100 solution for 20min. After Triton solution was removed, the brain slices were incubated overnight (12-16hrs) with mouse monoclonal anti-NPY (1:1000, ab112473, Abcam, Cambridge, UK) or mouse monoclonal anti-POMC (1:500, ab73092, Abcam, Cambridge, UK) primary antibodies at 4°C. Slides were then washed 3 times with PBS and secondary goat anti-mouse IgG (H+L) antibody, Alexafluor 555 (A-21422, Thermo Fisher Scientific, Massachusetts, USA) was added to the slides and incubated for 4hrs at 4°C. After incubation, the slides were washed with PBS and rabbit monoclonal anti-NeuN (1:1000, ab177487, Abcam, Cambridge, UK) or rabbit polyclonal anti-c-Fos (1:2000, ab190289, Abcam, Cambridge, UK) primary antibodies were introduced onto the slides and incubated overnight (12-16hrs) at 4°C. Slides were washed 3 times and a secondary goat anti-rabbit IgG (H+L) antibody, Alexafluor 488 (A-11008, Thermo Fisher Scientific, Massachusetts, USA) was added and incubated for 4hrs at 4°C. After incubation, slides were washed for a final time and mounted with Lab Vision™ Permafluor™ Aqueous Mounting Medium and coverslips were applied.

**Imaging and Quantification**

Fluorescent microscopy was performed using the Zeiss Axio Scope A1 equipped with an Axiocam 506 high resolution camera and a Photometrics Evolve 512 EMCCD high sensitivity camera. Fluorescence was detected by using FITC (488nm) and TRITC (543nm) filters to detect Alexafluor488 and Alexafluor555, respectively. CZI files were acquired using the Zen software
(Carl Zeiss, Oberkochen, Germany), which was converted to TIFF files and analyzed using Image J (NIH, Maryland, USA) and Image Pro Plus (version 4.5.; Media Cybernetics, Inc.) software. Images were separated into independent RGB channels using Zen software, where FITC signals merged with TRITC signals to display double-labeled cells and analysis of NPY/POMC fluorescence with NeuN/c-Fos fluorescence. Brightness and contrast of final images (1024x1024 pixels) were adjusted using Zen software.

For quantification procedures, the entire ARC was captured in 4 images and a total of 4 consecutive coronal sections were imaged from each animal. The average number of neurons from the 4 sections was treated as a single data value for each rat. A total of 3-6 rats were included in each treatment group for statistical analyses. All cell counting was done double-blinded to the treatment groups. All neurons in the ARC were counted using a 20x objective lens. Due to the underdeveloped brain at birth in the Wistar rat model, the ARC was not yet formed [223]. Therefore, images of brain sections at birth included the entire hypothalamus, while images taken at 9-weeks post-weaning captured the ARC only. To determine the expression of Pomc and Npy genes in in situ hybridization, cells were automatically counted by using the Image J Cell Counter option (http://rsbweb.nih.gov/ij/), based on density of immunofluorescence emitted from the cells. To quantify the immunohistochemistry slides, the number of co-labeled NPY/ POMC and NeuN/c-Fos positive neurons in the ARC were counted manually and reported as a percentage out of total single-labeled NPY or POMC containing neurons.
**Statistical Analysis**

A two-way repeated measures analysis of variance (ANOVA) using the PROC MIXED procedure in SAS (Version 9.4, SAS Institute Inc., Carey, NC, USA) was used to analyze body weight and weekly food intake calculated as g/day from weaning to termination (9-weeks post-weaning). Body weight data was log transformed to correct for normality. Diet and time were chosen as main factors in this model, including a diet*time interaction term. Non-significant interaction terms were removed from the final model. The effect of diet on body weight at birth and at weaning, and 5-MTHF levels in the hypothalamus at birth and at 9-weeks post-weaning, were analyzed by a one-way ANOVA. A one-way ANOVA was also used to assess data obtained from *in situ* hybridization and immunohistochemistry. Expression of NPY and POMC positive immunostaining as well as double-labeled NPY/POMC with NeuN/c-Fos neurons were evaluated separately at birth and at 9-weeks post-weaning. In the event of a significant interaction or main effect, a Tukey-Kramer post-hoc multiple comparison analysis was conducted. P<0.05 was considered statistically significant. All data were expressed as mean ± standard error of the mean (SEM).

**4.4 Results**

**Body Weight and Food Intake**

At birth, body weight was lowest in male offspring of dams fed the RF diet (control) compared to all other treatment groups (0RF 6.71±0.06g, RF 6.33±0.38g, 2.5RF 6.70±0.09g, 5RF 6.73±0.09g, 10RF 6.71±0.10g; p<0.05, **Fig. 4-2**). Offspring of dams fed either low or high doses of gestational folic acid averaged 20% greater body weight at 9-weeks post-weaning than the RF
group (Diet p=0.0017, Time p<0.0001, Diet*Time p=0.14, Fig. 4-3). From 5-weeks post-weaning until 8-weeks post-weaning, weekly food intake was significantly greater in all treatment groups (0RF, 2.5RF, 5RF, 10RF) compared to the control group (RF) (p<0.05, Fig. 4-4). Weekly food intake of male offspring was consistent with overall body weight results where offspring of dams fed the RF diet showed a ~9.7% reduction in food intake compared to each treatment group at 9-weeks post-weaning. (Diet p=0.0002, Time p<0.00001, Diet*Time p<0.0001). Cumulative food intake from 0-9 weeks post-weaning was ~11% lower in the RF group compared to all other treatment groups. (0RF 232.52±17.13g, RF 208.09±21.77g, 2.5RF 231.43±23.12g, 5RF 232.71±13.01g, 10RF 235.87±18.62g; p<0.05, Fig. 4-4 Inset)

**Figure 4-2. Body weight (g) of male offspring at birth.** Diet abbreviations: AIN93G with: RF(recommended folic acid) diet = 2mg/kg folic acid; 0RF = 0mg/kg (no added) folic acid, 2.5RF = 5mg/kg folic acid, 5RF = 10mg/kg folic acid, 10RF = 20mg/kg folic acid. \(^{ab}\) P<0.05 by one-way ANOVA followed by Tukey’s post-hoc analysis. Values are mean±SEM, n=36-43/group.
Figure 4-3. Body weight (g) of male offspring from 0-9 weeks post-weaning. Diet abbreviations: AIN93G with: RF(recommended folic acid) diet = 2mg/kg folic acid: 0RF = 0mg/kg (no added) folic acid, 2.5RF = 5mg/kg folic acid, 5RF = 10mg/kg folic acid, 10RF = 20mg/kg folic acid. Body weight: diet p=0.0017, time p<0.0001. \(^{ab}\) P<0.05 by PROC MIXED model repeated measures followed by Tukey’s post-hoc analysis. Non-significant diet*time interaction term (p=0.14) was removed from the model shown here. Values are mean±SEM, n=11-14/group.
Figure 4-4. Weekly food intake of male offspring from 0-9 weeks post-weaning. Diet abbreviations: AIN93G with: RF(recommended folic acid) diet = 2mg/kg folic acid: 0RF = 0mg/kg (no added) folic acid, 2.5RF = 5mg/kg folic acid, 5RF = 10mg/kg folic acid, 10RF = 20mg/kg folic acid. Diet p=0.0002, time p<0.0001, diet*time interaction p<0.0001. *ab P<0.05 by PROC MIXED model repeated measures followed by Tukey’s post-hoc analysis. [Inset: Cumulative food intake at 9-weeks post-weaning. ab P<0.05 by one-way ANOVA followed by Tukey’s post-hoc analysis.] Values are mean±SEM, n=11-14/group.

5-MTHF Concentrations in the Hypothalamus

5-MTHF levels were analyzed in the hypothalamus of pups at birth to determine its response to the different folic acid diets consumed during pregnancy. 5-MTHF is the only bioavailable form of folate found in the brain and is the most susceptible to gestational folic acid supplementation [308].
Offspring of dams fed the RF diet exhibited a lower concentration of 5-MTHF in the hypothalamus compared to the 5RF, 10RF, and 0RF groups, but not the 2.5RF group (p<0.05, Fig. 4-5A). A significant dose-dependent increase was observed in offspring born to dams from the RF group to those given higher folic acid supplementation (5RF and 10RF). 5-MTHF concentration in the hypothalamus of the 5RF and 10RF group were 49.9% and 66.6% higher than the RF group, respectively (p<0.05). In contrast, the offspring of dams who were fed a 0RF (no added folic acid) gestational diet had the highest accumulation of 5-MTHF in the hypothalamus with a concentration 75.4% greater than the RF group (p<0.05). At 9-weeks post-weaning, no differences in 5-MTHF levels in the hypothalamus were found across all groups (p=0.9293, Fig. 4-5B)

![Figure 4-5. 5-Methyltetrahydrofolate concentration (ug/g protein) in the hypothalamus of male offspring at (A) birth and (B) 9-weeks post-weaning.](image)

The quantified weight of 5-MTHF is expressed relative to hypothalamus protein weight (ug/g). Diet abbreviations: AIN93G with: RF (recommended folic acid) diet = 2mg/kg folic acid; 0RF = 0mg/kg (no added) folic acid, 2.5RF = 5mg/kg folic acid, 5RF = 10mg/kg folic acid, 10RF = 20mg/kg folic acid. a,b,c,d P<0.05 by one-way ANOVA followed by Tukey’s post-hoc analysis. Values are mean±SEM, n=4-5/group.
Pomc and Npy Gene Expression at Birth and 9-weeks Post-weaning

To determine the relationship between gestational folic acid content and gene expression of food intake regulatory markers, we examined expression of Npy and Pomc genes in neuronal cells at birth and 9-weeks post-weaning using in situ hybridization technique. The expression of Npy and Pomc genes in the hypothalamus of offspring at birth was not affected by either low or high doses of gestational folic acid, (Npy: p=0.9910, Pomc: p=0.8185, Fig. 4-6).

Figure 4-6. Fluorescence in-situ hybridization of Npy (red) and Pomc (green) genes in the hypothalamus of male offspring at birth. Expressing (A) Npy and (B) Pomc, identified by the arrows, were counted according to protocol described in the Methods section. Each group represents the average counts of 4 coronal sections per rat, specifically of the hypothalamus only. Diet abbreviations: AIN93G with: RF(recommended folic acid) diet = 2mg/kg folic acid: 0RF = 0mg/kg (no added) folic acid, 2.5RF = 5mg/kg folic acid, 5RF = 10mg/kg folic acid, 10RF = 20mg/kg folic acid. P>0.05 by one-way ANOVA model. Values expressed as mean±SEM, n=3/group. Scale bars: A,B, 20µm (20x magnification).
Although overall cell count increased in later life compared to the levels expressed at birth, gestational folic acid content did not affect \textit{Npy} and \textit{Pomc} gene expression in the ARC at 9-weeks post-weaning. (\textit{Npy}: \(p=0.3870\), \textit{Pomc}: \(p=0.7331\), Fig. 4-7).

Figure 4-7. Fluorescence \textit{in-situ} hybridization of \textit{Npy} (red) and \textit{Pomc} (green) genes in the ARC of male offspring at 9- weeks post-weaning. Expressing (A) \textit{Npy} and (B) \textit{Pomc}, identified by the arrows, were counted according to protocol described in the Methods section. Each group represents the average counts of 4 coronal sections per rat, specifically of the ARC only. Diet abbreviations: AIN93G with: RF(recommended folic acid) diet = 2mg/kg folic acid: 0RF = 0mg/kg (no added) folic acid, 2.5RF = 5mg/kg folic acid, 5RF = 10mg/kg folic acid, 10RF = 20mg/kg folic acid. \(P>0.05\) by one-way ANOVA model. Values expressed as mean±SEM, \(n=3\)/group. Scale bars: A,B, 20µm (20x magnification).
**POMC and NPY Neuron Maturation and Activity at Birth in the Hypothalamus**

To further examine the *in utero* effects of gestational folic acid content on the food intake regulatory pathways, we investigated the protein expression of NPY and POMC positive neurons as well as their neuronal maturation and activity at birth using immunohistochemistry.

Folic acid content of the gestational diet affected protein expression of NPY-positive neurons in the hypothalamus of the pups at birth. The percentage of mature double-labeled NeuN+ NPY+ neurons out of the total number of NeuN-labeled neurons in the hypothalamus was highest in the 5RF group compared to all other groups (p<0.05, 0RF=1.3%, RF=2.3%, 2.5RF=2.4%, 5RF=8.2%, 10RF=2.4%, [Fig. 4-8D]). Furthermore, in each dietary group, more than 96% of NPY+ neurons had reached maturation at birth (NPY expression ≥96.1%; p=0.6360; [Fig. 4-8E]). Gestational folic acid content was not found to affect NPY+ neuronal activity at birth, as less than 1% of NPY+ neurons in the hypothalamus expressed c-Fos activity (p=0.8757, [Fig. 4-8F]).

Contrary to NPY expression, gestational folic acid content did not affect expression, maturation, or activation of POMC+ neurons in the hypothalamus at birth (p>0.05). NeuN+ POMC+ neurons in the hypothalamus of rat offspring at birth (p=0.7, [Fig. 4-8J]) were not affected, as 98.8% of the POMC+ neurons in the hypothalamus were already mature at birth (p=0.4662, [Fig. 4-8K]). In addition, less than 5% of the POMC+ neurons at birth expressed c-Fos activity (p=0.6332, [Fig. 4-8L]).
Figure 4-8. Immunofluorescence staining of expression, maturation, and activation of NPY and POMC neurons in the hypothalamus of male offspring at birth. Each group represents the average counts of 4 coronal sections per rat, specifically of the hypothalamus. Representative immunofluorescence staining of (A) NPY (red), (G) POMC (red), (B,H) NeuN (green), (C) co-labeling of NPY+NeuN+, and (I) co-labeling of POMC+NeuN+ neurons in the hypothalamus. Arrowheads point to respective hypothalamic positively labeled neurons. Quantifications of NPY-positive neurons: (D) Percent of double-labeled NeuN+NPY+ neurons out of total NeuN-labeled cells in the hypothalamus. (E) Percent of NPY+ neurons expressing NeuN. (F) Percent of NPY+ neurons expressing c-fos. Quantifications of POMC-positive neurons: (J) Percent of double-labeled NeuN+POMC+ neurons out of total NeuN+ cells. (K) Percent of POMC+ neurons expressing NeuN. (L) Percent of POMC+ neurons expressing c-fos. Diet abbreviations: AIN93G with: RF(recommended folic acid) diet = 2mg/kg folic acid: 0RF = 0mg/kg (no added) folic acid, 2.5RF = 5mg/kg folic acid, 5RF = 10mg/kg folic acid, 10RF = 20mg/kg folic acid. \(^{ab}\) P<0.05 by one-way ANOVA model and Tukey’s post-hoc analysis. Values expressed as mean±SEM, n=5-6/group. Scale bars: A,B,C,G,H,I 50µm (20x magnification).

POMC and NPY Neuron Activity at 9-weeks Post-weaning in the ARC

At 9-weeks post-weaning, the ARC of the mature male offspring were isolated and quantified to determine the effects of gestational folic acid content on NPY and POMC food intake regulatory pathways in mature rats. In contrast to at birth, no differences between groups were observed in the number of NPY+ expressing neurons in the ARC (p=0.8124, Fig. 4-9D). NPY+ activity was similar across all the dietary groups at 9-weeks post-weaning (p=0.4286, Fig. 4-9E). Out of the total number of NPY+ neurons expressed in the ARC, only 11.4-17.0% of these neurons among all the dietary groups, were activated and co-labeled with c-Fos immunostaining.

The expression of POMC+ neurons in the ARC of mature Wistar rat offspring at 9-weeks post-weaning was not different between dietary groups (p=0.1110, Fig. 4-9I). However, activation of c-Fos+ POMC+ expressing neurons was 32.4-50.5% higher in the control (RF) group than in the 2.5RF, 5RF, and 10RF groups (p<0.05, Fig. 4-9E).
Figure 4-9. Immunofluorescence staining of expression and activation of NPY and POMC neurons in the ARC of male offspring at 9-weeks post-weaning. Each group represents the average counts of 4 coronal sections per rat, specifically of the hypothalamus. Representative immunofluorescence staining of (A) NPY (red), (F) POMC (red), (B,G) c-fos (green), (C) co-labeling of NPY+c-fos+, and (H) co-labeling of POMC+c-fos+ neurons in the hypothalamus. Arrowheads point to respective hypothalamic positively labeled neurons. Quantifications of NPY-positive neurons: (D) The number of NPY+ neurons in the ARC. (E) Percent of NPY+ neurons expressing c-fos. Quantifications of POMC-positive neurons: (I) Cell count of POMC+ neurons in the ARC. (J) Percent of POMC+ neurons expressing c-fos. Diet abbreviations: AIN93G with: RF(recommended folic acid) diet = 2mg/kg folic acid: 0RF = 0mg/kg (no added) folic acid, 2.5RF = 5mg/kg folic acid, 5RF = 10mg/kg folic acid, 10RF = 20mg/kg folic acid. P<0.05 by one-way ANOVA model and Tukey’s post-hoc analysis. Values expressed as mean±SEM, n=5-6/group. Scale bars: A,B,C,F,G,H 50µm (20x magnification).

4.5 Discussion

Through this study we have shown that gestational folic acid below and above requirements alter the development of food intake neurons in the hypothalamus which may favour higher food intake and body weight in the offspring post-weaning. It is the first to show that intakes of folic acid, both below and above that recommended, during pregnancy affected the maturation, expression and function of hypothalamic food intake regulatory pathways, as measured in NPY and POMC neurons. Moreover, both low and high folic acid gestational diets, compared with the RF diet, resulted in higher birth weight, and higher post-weaning body weight and food intake in the offspring. However, the relationship between the effects of the gestational diets on NPY- and POMC-expression neurons and on food intake of the offspring is unclear.

Low (0RF), moderately high (2.5RF, 5RF) and very high (10RF) levels of folic acid fed during pregnancy resulted in higher body weight of male Wistar rat offspring from birth to post-weaning compared to recommended levels (RF). These effects of the higher folic acid
gestational content are consistent with previous studies that have reported higher birth weight [81,224,309-317]. However, this is the first study to show higher birth weight due to low folic acid gestational diet in male Wistar rat offspring, as previous studies have either shown no effect or low birth weights in offspring [64,159,318-320]. The similar birth weight between the 0RF and those above recommended levels, particularly the 10RF group, suggests a common in utero mechanism in which very low or high gestational folic acid affects birth weight.

As an indicator of the activity in the one-carbon metabolic pathway, 5-MTHF was measured in the hypothalamus. Its concentration increases linearly with increasing content of folic acid in the gestational diets, consistent with previous reports [8]. Distinctly, offspring born to dams fed 0RF had the highest concentration of 5-MTHF in the hypothalamus. Higher levels of 5-MTHF with increasing dietary folic acid intake may in part arise from suppression of the conversion of 5-MTHF into tetrahydrofolate (THF) [89,114]. As well, the conversion of 5,10-methylene-THF to 5-MTHF is irreversible, making a build-up of 5-MTHF plausible [108,120]. However, it is harder to explain the elevated concentrations arising from the 0RF gestational diet because the source of the 5-MTHF is unknown and previous literature have only linked folic acid supplementation but no evidence of deficiency increasing 5-MTHF levels in the brain of rodent offspring [336]. Nevertheless, these results show that folic acid intake during pregnancy greatly influence the availability of methyl donors in 1-carbon metabolism in the hypothalamus of the offspring.

Although the study results show that folic acid content of the gestational diet influences development of food intake regulation in the hypothalamus, the specific role of NPY and POMC in the regulation of food intake in the offspring remains unclear. Folic acid content of the gestational diets affected expression of mature NPY neurons in the hypothalamus, but no clear
relationship was evident between dose given and food intake or body weight of the offspring. Expression of NPY neurons was significantly greater in the 5RF supplemented group compared to all other diet groups; but all groups were heavier at birth and had higher food intake and body weight than the RF group, post-weaning.

In contrast to NPY neurons, no differences were found in the number of mature POMC neurons in the hypothalamus of offspring at birth. These findings were expected as overall POMC neurons have a delayed developmental pattern in the offspring [321]. Since both NPY and POMC neurons share the same progenitor cell line, POMC expression is suppressed in a large number of immature hypothalamic neurons during gestation to enable NPY neuronal maturation [223]. The orexigenic NPY neuronal population is favoured since early stages of life require rapid body growth and higher energy demands [181,197,322-324]. Similarly, no differences were observed in the number of active (as shown by c-Fos expression) NPY or POMC neuron populations, possibly since neuronal connectivity and activity is not well established until the second week of life in rodent models [195,214,215]. Although NPY and POMC neurons are derived from the same progenitor cell lineage, this study shows that these neuronal cell types develop and arise from the POMC-expressing lineage at distinct time points during gestation and even post-natal. In addition, gestational folic acid may influence in utero cell fate decisions within these immature neuronal populations, which could permanently affect the composition and function of feeding-related neurocircuits.

Consistent with our previous report, 10-fold intakes of gestational folic acid lead to higher body weight and food intake in male Wistar rat offspring post-weaning [8]. However, feeding dams a folic acid diet during gestation that was either below or in a range above recommended intakes induced similar phenotypic consequences in the post-weaning offspring. These results highlight
the importance of an optimal dose of folic acid during pregnancy and are consistent with a recent cohort study which showed an L-shaped association between maternal folate status and childhood over-weight or obesity [327].

Food intake and body weight of offspring was higher in those born to mothers fed the 0, 2.5, 5.0 and 10RF diets compared with those from dams fed the RF diet to 9-weeks of age. Due to the long-term plasticity of the hypothalamic food intake pathways that persist into adulthood, the differences observed at birth cannot explain post-weaning results. By 9-weeks post-weaning, the influence of gestational folic acid on the 5-MTHF levels in the hypothalamus were absent, possibly due to the long-term post-weaning consumption of the RF diet. Similar to the results seen at birth, neither Npy nor Pomo gene expression was affected by gestational diet folic acid content. Although this result contrast with a previous report that gestational intakes of 10-fold folic acid result in lower Pomo mRNA expression in the hypothalamus of mature male offspring post-weaning [8] measurement was of whole hypothalamic gene expression, rather than the specific ARC nuclei as done in the present study. In the present study, activity of POMC neurons was highest in those from dams fed the RF diet, consistent with their lower food intake and body weight. Thus, high intakes of folic acid during pregnancy may have altered maturation of POMC neurons post-birth, affecting long-term energy balance of the offspring via the function of POMC neurons.

By 8-weeks post-weaning, food intake in all treatment groups decreased up to termination, which may reflect an acclimatization mechanism due to the RF diet fed to the pups post-weaning, adjusting food intake regulation to maintain energy balance. The hypothalamic feeding pathways continue to be highly plastic throughout adulthood, potentially erasing the cellular and phenotypic changes that occurred at birth. This is supported by our previous study [8] showing
that when offspring from dams fed a 10-fold folic acid diet were given a high-folic acid diet post-weaning, they did not express the obesogenic phenotype and changes in \textit{Pomc} mRNA expression were corrected.

This study design had many strengths; \textit{in situ} hybridization and immunohistochemistry techniques allowed for quantitative and physical analysis of gene and neuronal expression and function of food intake markers, specifically targeting two key neuronal populations in the ARC, the feeding center of the brain. In addition, timed pregnant Wistar dams ensured control over the duration of treatments, and consistency of body weight and food intake measurements within each group. It was the first study to confirm that amounts lower than 10-fold recommendation of gestational folic acid produced significant differences in the hypothalamic food intake pathways and phenotype of male offspring; allowing the results to be more relevant to human populations. In developed countries, pregnant women are known to consume doses 2-7 times greater than recommended levels, comparable to the doses incorporated in the AIN-93G diet in this study [7,87].

However, the mechanisms by which these diets had their effects remain unclear. While the ARC contains the majority of feeding-related neurons, changes in the ARC alone are unlikely to fully explain the differences observed in body weight and food intake later life. Future studies must explore second-order neurons as well as other key food intake regulatory neuropeptides to better understand the mechanisms mediating folic acid induced changes in the food intake pathways. Further research must also be done to obtain a clearer map of cell fate in the hypothalamus during development due to different levels of folic acid. Investigation of 1-carbon metabolite induced changes by low and high folic acid gestational diets at birth, as well as epigenetic mechanisms will be required to provide more insight towards the molecular mechanisms of
gestational folic acid induced changes in central energy balance pathways.

The findings from this research have potential application in human health. By investigating the *in utero* neurodevelopment of offspring in an animal model, we have shown that deviations from requirements, both high and low, have potential adverse effects on development of metabolic phenotype and central food intake regulatory pathways in offspring. Along with childhood obesity trends in North America, we have seen an increased consumption of vitamins in women [78,328]. Due to the risk of neural tube defects in newborns, folic acid intake during pregnancy has increased beyond the tolerable upper limit levels (UL, >1000μg/day, 2.5-7 fold above recommended level) over recent years [3,4,7,87]. The Canadian Health Measures Survey from 2007-2009 reported folate deficiency to be virtually nonexistent in the Canadian population and high blood folate concentrations were evident in 40% of the female participants at child bearing age [5]. In turn, the offspring is exposed to high levels of folic acid during early development. This raises a possible concern and relationship between gestational folic acid consumption and unknown long-term consequences [88].

### 4.6 Conclusion

Folic acid content in the gestational diet affects development of food intake regulatory pathways at birth and in adulthood, which may explain higher food intake and body weight in male Wistar rat offspring post-weaning.
CHAPTER 5

Summary and Future Directions

The results from the present study demonstrate that low and high doses (0RF, 2.5RF, 5RF, and 10RF) of folic acid provided in utero induced dose-related changes on expression and activity of food intake regulatory neuron populations at birth and later life in the offspring. These changes in the hypothalamic food intake regulatory pathways were supported by increased body weight at birth that persisted into adulthood.

As the first study to examine the effects of gestational folic acid on the development and function of the POMC and NPY neurons at birth and at adulthood, this study proposes improvements and future directions. In the present study, we chose the Wistar rat model (an outbreed strain with genetic diversity) to explain the effects of gestational folic acid content on development of hypothalamic food intake pathways. There are many advantages in choosing this model, including their short gestation period and lifespan as well as their similar dietary patterns to humans. Most importantly, rats share the same neurodevelopmental trajectory as humans [2]. However, timing of developmental processes differs between rats and humans [330]. Rats, classified as altricial species, are relatively immature at birth compared to humans. The neurodevelopment of a rat at birth equates to that of a human fetus in the third trimester [195]. Therefore, future studies should examine the hypothalamic food intake pathways of offspring one week after birth, between post-natal days 6-10 [196] which correlates to the development of the human brain at birth.

Although this study looked at the development of hypothalamic food intake pathways at birth and adulthood, it could not explain the development that occurred within the gestation period. In
order to examine maternal folic acid effects in gestation, offspring must be terminated for brain tissue collection during embryogenesis. This study design would require a greater number of dams for a subset to be terminated once embryos are obtained during pregnancy. In addition, to obtain rat fetuses of a known gestational age, a further set of fertile females and males will be required for timed mating. Although this protocol will generate further costs due to an increased sample size as well as the need to order fertile males, it does not pose any harm to the mother or the offspring.

An alternative method to evaluate development within gestation is by using 5-Bromo-2’-deoxyuridin (BrdU) labeling. Although this method does not require any additional Wistar dams, embryonic BrdU treatment is known to pose many detrimental side effects on cell cycle, differentiation and survival, which may affect the development of feeding-related neurons in the offspring [331]. BrdU has also resulted in mothers yielding offspring with reduced brain and body weight [332].

The role that neurotrophic hormones play during fetal development remains critical. In particular, leptin directly regulates the development and activity of food intake regulatory pathways within the ARC as well as postnatal regulation of food intake and body weight, which was not investigated in this study [195,196]. Leptin appears to act primarily during a restricted neonatal critical period, known as a leptin surge. In turn, daily post-natal monitoring of leptin levels in the brain would be required throughout the lactation period in order to detect the leptin surge. This could not be done in the current protocol due to the limited litter sizes, where male Wistar rat offspring were needed for in situ hybridization and immunohistochemistry. However, knowledge of the timing and amplitude of the post-natal leptin surge would be most valuable to the understanding of how gestational folic acid may affect leptin, which consequently modulates
neurodevelopment of arcuate feeding-related pathways and long-term body weight regulation as well as adiposity in the offspring.

Furthermore, Wistar dams were fed different doses of folic acid during pregnancy and were all switched to the recommended folic acid diet during lactation. However, in human populations, high maternal folic acid intakes continue to persist throughout lactation, exposing the infant to elevated levels of folates after birth through milk consumption [6,50]. By keeping the Wistar mothers on their folic acid treatment diets during lactation up to weaning, we can mimic more closely the maternal folic acid consumption patterns and folate exposure in children during early life of human populations. As a result, future studies should exhibit how the effects of folic acid, below and above requirements, provided in utero and during lactation affect the development of the hypothalamic feeding-related pathways of offspring at birth, weaning, and later in life.

Despite measuring concentrations of 5-MTHF in the hypothalamus, further investigation of substrates involved in methyl group transfers is crucial, specifically the SAM:SAH ratio. By determining the levels of SAM and SAH in the hypothalamus, we will be able to obtain a better understanding of the increased levels of 5-MTHF observed in the 0RF and 10RF groups at birth and confirming a potential block in 1-carbon metabolism. In addition, since the SAM:SAH ratio is known to regulate DNA methylation, we will be able to predict the effects of gestational folic acid on methionine synthesis in the hypothalamus through the availability of SAM and SAH, a product of the methylation reaction [115,333].
Lastly, through this study we examined feeding-related neuronal populations within the ARC in later life. However, based on the results seen from *in situ* hybridization and immunostaining of *NPY* and *POMC*, the effects of gestational folic acid may extend beyond the ARC into second-order neurons in the hypothalamus. Therefore, future research should investigate projections emanating from the ARC, including the paraventricular nucleus, lateral hypothalamus, dorsomedial hypothalamus and ventromedial hypothalamus. By understanding the full impact of gestational folic acid on the neurodevelopment of offspring using the rat model, we can potentially deduce adverse effects on the food intake regulatory pathway as well as the onset of childhood obesity in offspring of pregnant women in Canada who consume folic acid well above the established UL.
References

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Appendices
Appendix 1. Summary of animal and human studies on the effect of high vitamin or folic acid intakes and offspring obesity.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Model</th>
<th>Maternal Diet and Duration of Treatment</th>
<th>Effect on Offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pannia et al. (2015) Behav Brain Res, 278:1-11</td>
<td>Animal Wistar Rat</td>
<td>Pregnancy (3 weeks): 10-fold multivitamin (incl. folic acid) diet. 10-fold multivitamin diet with recommended folic acid (2mg/kg diet). 10-fold methyl vitamin (folic acid, B12, B6) diet. Post-weaning (15 weeks): High fat (60%) lard diet. AIN-93G diet (2mg/kg folic acid)</td>
<td>Dams on 10-fold multivitamin diet gave birth to male offspring with: ↑ Post-weaning weight gain. ↑ Leptin and insulin signalling genes (hypothalamus). Dams on 10-fold multivitamin diet with recommended level of folic acid gave birth to male offspring with: - Modulated weight gain and gene expression. Dams on 10-fold methyl vitamin diet gave birth to male offspring with: ↑ Post-weaning weight gain.</td>
</tr>
<tr>
<td>Study</td>
<td>Animal</td>
<td>Diet Details</td>
<td>Offspring Effects</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>--------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Carlin et al. (2013) PLoS One, 8:e63549</td>
<td>Animal C57BL/6 Mouse</td>
<td>Pregnancy + Lactation (6 weeks): High fat + high methyl vitamin diet High methyl vitamin incl.: 3-9 fold folic acid, 20-60 fold B12, 3-9 fold choline, 3-9 fold betaine Post-weaning (50 weeks): Control diet</td>
<td>Dams gave birth to male offspring with: ↑ Weight gain ↑ Fat preference</td>
</tr>
<tr>
<td>Cho et al. (2015) Mol Nutr Food Res, 59: 476-89</td>
<td>Animal Wistar Rat</td>
<td>Pregnancy (3 weeks): 10-fold methyl vitamin (folic acid, B12, B6) diet Post-weaning (8 weeks): High fat (60%) 1rd diet AIN-93G diet (2mg/kg folic acid)</td>
<td>Dams gave birth to male offspring with: ↑ Body weight ↑ Food intake ↑ Adiposity ↑ Metabolic hormones ↑ Sucrose preference ↓ POMC mRNA in hypothalamus associated with increased DNA methylation</td>
</tr>
<tr>
<td>Cho et al. (2013) Int J Obes, 37 (9): 1177-82</td>
<td>Animal Wistar Rat</td>
<td>Pregnancy (3 weeks): 10-fold multivitamin (incl. folic acid) diet Post-weaning (28 weeks): 10-fold folic acid diet AIN-93G diet (2mg/kg folic acid)</td>
<td>Dams gave birth to male offspring with: ↑ Body weight ↑ Food intake ↓ POMC</td>
</tr>
<tr>
<td>Huang et al. (2014) Int J Mol Sci, 15(4):6298-313</td>
<td>Animal C57BL/6 Mouse</td>
<td>Pregnancy (3 weeks): 2.5-fold folic acid diet 8-fold folic acid diet Post-weaning (12-48 weeks): High fat (15%) diet AIN-93G diet (2mg/kg folic acid)</td>
<td>Dams gave birth to male offspring with: ↑ Weight gain ↑ Food intake ↑ Glucose/insulin intolerance ↑ Global DNA methylation in adipose</td>
</tr>
<tr>
<td>Reference</td>
<td>Study Design</td>
<td>Maternal Diet and Duration of Treatment</td>
<td>Effect on Offspring (male and female)</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-------------------------</td>
<td>----------------------------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| Changamire et al. (2012) Matern Child Nutr | RCT 8468 women in Tanzania | Multivitamin + additional folic acid supplementation (vitamin B1 (20mg), B2 (20mg), B3 (100mg), B6 (25mg), B12 (0.5mg), C (500mg), E (30mg), folic acid (800+250µg)) | Offspring showed:  
↑ Mean birth weight                                                                                   |
| Asemi et al. (2014) Int J Prev Med, 4:439-46 | Single-blind RCT 104 women in Iran | Multivitamin/multimineral supplementation (vitamin B1 (1.5mg), B2 (1.7mg), B3 (20mg), B6 (2mg), folic acid (400µg), B12 (6µg), A (3000µg), E (13.5µg), D (10µg), C (60mg), calcium (250mg), iron (27mg), zinc (25mg)  
20 weeks starting at 16 weeks gestation | Offspring showed:  
↑ Mean birth weight  
↑ Head circumference                                                                                   |
| Yajnik et al. (2008) Diabetologia, 51:29-38 | Human Prospective Cohort 700 women in India | Maternal micronutrient and status collected at 18 and 28 weeks of gestation  
6 year follow up on 653 children                                                                 | During gestation, low maternal vitamin B12 and high folate concentration was associated with offspring with:  
↑ Adiposity  
↑ Insulin resistance                                                                                   |
| Wang et al. (2016) JAMA Pediatr, 170(8):p.e160845 | Human Prospective Cohort 1517 mothers in USA | 9 year follow up                                                                                                                                                  | Offspring had L-shaped association between maternal folate concentrations and child body mass index and metabolic biomarkers.  
↑ Risk with lowest and highest quartile of folates.                                                   |
Appendix 2. Composition of vitamin mix in experimental diets (V10037).

<table>
<thead>
<tr>
<th>Vitamin (g/kg diet dry weight)</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine HCL (Vitamin B1)</td>
<td>6 mg</td>
</tr>
<tr>
<td>Riboflavin (Vitamin B2)</td>
<td>6 mg</td>
</tr>
<tr>
<td>Pyridoxine HCL (Vitamin B6)</td>
<td>7 mg</td>
</tr>
<tr>
<td>Niacin (Vitamin B3)</td>
<td>30 mg</td>
</tr>
<tr>
<td>Folic acid (Vitamin B9)</td>
<td>2 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Cobalamin (0.1 %, Vitamin B12)</td>
<td>25 μg</td>
</tr>
<tr>
<td>Vitamin A palmitate</td>
<td>4,000 IU</td>
</tr>
<tr>
<td>Vitamin E acetate</td>
<td>75 IU</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>1000 IU</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin K1</td>
<td>0.75 mg</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>16 mg</td>
</tr>
</tbody>
</table>

**Note:** V14905 is the same as V10037 with no folic acid (Vitamin B9).
Appendix 3. Sample size determination.

Statistical requirement to have each group of male Wistar rat as an independent unit for statistical analysis.

Based on one-way ANOVA

\[ \alpha = 0.05 \]

\[ \beta = 0.8 \]

\[ N = 10/\text{group} \] to detect a 10% effect size on body weight [152].

\[ N = 3/\text{group} \] to detect differences in \textit{in situ} gene expression [223] and immunohistochemistry protein expression [195].