ABSTRACT

The Effect of Dietary Folate Deficiency and Age on Methylation Metabolites, Neurotransmitters, and Behavior in Mice

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Severe folate deficiency caused by inborn errors of metabolism has profound deleterious effects in the CNS such as mental retardation, psychiatric disorders, seizures and myelopathy. Mild folate deficiency, due to dietary insufficiency, drugs, or a common mutation in the gene encoding methylenetetrahydrofolate reductase (MTHFR C677T), are associated with an increased risk for depression and dementia, especially in the aging population. The metabolism of folate is intimately linked with the synthesis of S-adenosylmethionine (SAM), the sole source of methyl groups required in methyltransferase reactions. S-adenosylhomocysteine (SAH), a product of methylation reactions, accumulates in folate deficiency due to increased conversion from homocysteine. Recent studies have linked hyperhomocysteinemia and hypomethylation to gene activity, as well as methylation dependent post-translational modification of proteins and neurotransmitter metabolism in depression and dementia.

To better define the role of folate deficiency in CNS function, we fed young and old C57BL/6J mice folate deficient diets for 3 months and old heterozygous *tg*-MTHFR mice a low folate diet for 6 months. Mice were tested for grip strength,

coordination, open field activity, and spatial memory. After treatment mice were sacrificed by CO_2 asphyxiation or microwave radiation. Blood, peripheral and regional brain tissues were processed for the analysis of methylation and neurotransmitter metabolites.

Age did not influence brain methylation metabolites in C57BL/6J mice. Low folate and folate deficient diets decreased the SAM/SAH ratio, an indicator of methylation status in most brain regions from C57BL/6J and heterozygous *tg*-MTHFR mice. Overall, the effects of folate deficiency were not exacerbated by age in C57BL/6J mice. Brain methylation metabolites differed significantly depending on the method of sacrifice. Specifically, folate deficiency decreased SAM in mice sacrificed by microwave radiation and increased SAH in mice sacrificed by asphyxiation. Folate deficiency reduced dopamine and serotonin turnover in several brain regions although the levels of the parent neurotransmitters were unaffected. Choline and acetylcholine levels were reduced by folate deficiency in the mid-brain. Folate deficiency impaired open field behavior, but did not have any significant effect on spatial memory. These studies help to further our understanding of the mechanisms involved in folate deficiency on CNS function in the aging brain.

The Effect of Dietary Folate Deficiency and Age on Methylation Metabolites, Neurotransmitters, and Behavior in Mice

by

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A Dissertation

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3-MT	3-methoxytyramine
5-HIAA	5-hydroxyindole acetic acid
5-HT	serotonin
5-HTP	5-hydroxytryptophan
5-MTHF	5-methyltetrahydrofolate
AADD	amino acid defined diet
Ach	acetylcholine
ACN	acetonitrile
AD	Alzheimer's disease
ADE	adenosine
ADMA	asymmetric dimethylarginine
ANOVA	analysis of variance
APP	amyloid precursor protein
ARG	arginine
ATP	adenosine triphosphate
BACE	β secretase
BBB	blood-brain barrier
BER	base excision-repair
BET	betaine
BHMT	betaine homocysteine methyltransferase
BH_4	tetrahydrobiopterin

CB	cerebellum
CBS	cystathionine β-synthase
CD	amino acid defined control diet
CE	collision energy
CFD	cerebral folate deficiency
ChAT	choline acetyltransferase
CHO	choline
CNS	central nervous system
CO_2	carbon dioxide
COMT	catechol-O-methyltransferase
CpG	cytosine-phosphate-guanidine
CSF	cerebral spinal fluid
CSL	cystathionine-γ-lyase
CX	cortex
CXP	collision exit potential
CYS	cysteine
CYSTA	cystathionine
DA	dopamine
DAT	dopamine transporter
DDAH	dimethylarginine dimethylaminohydrolase
DDC	amino acid decarboxylase
DETAPAC	diethylenetriaminepentaacetic acid
DHF	dihydrofolate

DHFR	dihydrofolate reductase
DHPR	dihydropteridine reductase
DHPS	dihydropteroate
DMF	dimethylformamide
DOPAC	3,4-dihydroxyphenylacetic acid
DTE	1,4-dithioerythritol
dTMP	deoxythymidine monophosphate
DTT	dithiothreitol
dUMP	deoxyuridine monophosphate
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
EP	entrance potential
ESAA	excitatory sulfur amino acids
EtOH	ethanol
FCX	frontal cortex
FD	amino acid defined folate deficient diet
FP	floor plane
FRs	folate receptors
GABA	γ-aminobutyric acid
GLN	glutamine
GLU	glutamate
GPx	glutathione peroixdase
GRed	glutathione reductase

GSH	glutathione
GSSG	oxidized glutathione
GTP	guanidine triphosphate
GTPCH	guanidine triphosphate cyclohydrolase
H_2O_2	hydrogen peroxide
HCA	homocysteic acid
HCSA	homocysteine sulfinic acid
Hcy	homocysteine
HHcy	hyperhomocysteinemia
HIP	hippocampus
HVA	3-methoxy-4-hydroxy-phenylacetic acid (homovanillic acid)
L-DOPA	levodopa
LF	amino acid defined low folate diet
MAO	monoamine oxidase
MAT	methionine adenosyltransferase
MB	mid-brain
MBP	myelin basic protein
MeOH	methanol
MET	methionine
MMSE	mini-mental state exam
MRM	multiple reaction monitoring
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthetase

MTRR	methionine synthetase reductase
MWM	Morris water maze
NAC	N-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NaOH	sodium hydroxide
NCD	normal chow diet
NE	norepinephrine
NHANES	national health and nutrition examination survey
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NTD	neural tube defect
OH	hydroxyl radicals
OSA	octyl sodium sulfate
PC	phosphatidylcholine
PCA	perchloric acid
PCFT	proton-coupled folate transporter
PCR	polymerase chain reaction
PD	Parkinson’s disease
PE	phosphatidylethanolamine
PEMTs	phospholipid methyltransferase enzymes
PP2A	protein phosphatase 2A
PRMT	protein arginine methyltransferase
PS1	presenilin 1

p-Tau	phospho-Tau
PTPS	6-pyruvoltetrahydrobiopterin synthetase
qBH_4	quinoid dihydrobiopterin
RBC	red blood cell
RFC	reduced folate carrier
ROS	reactive oxygen species
SAH	S-adenosylhomocysteine
SAHase	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
SBDF	7-fluoro-2,1,3-benzoxaazole-4-sulfonamide
SDMA	symmetric dimethylarginine
SHMT	serine hydroxymethyltransferase
SNPs	single nucleotide polymorphisms
SOD	superoxide dismutase
SR	sepiaterin reductase
SSA	succinylsulfathiazole
STR	striatum
TCA	trichloroacetic acid
tg-MTHR	transgenic MTHFR mice
TH	tyrosine hydroxylase
tHcy	total homocysteine
THF	tetrahydrofolate
TNBP	tri-*n*-butylphosphine

TPH	tryptophan hydroxylase
TRP	tryptophan
TS	thymidylate synthetase
TYR	tyrosine
UNG	uracil-DNA *N*-glycosylate
VP	vertical plane

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To my loving family and friends who have supported me financially and emotionally throughout these years

CHAPTER ONE

Introduction

Folate Derivatives and Transport

Folate

Pteroylmonoglutamate better known as folate is a water-soluble B vitamin that plays a major role in central nervous system (CNS) development and brain function by acting as a carrier of one-carbon units (Scott and Wire 1998). Folate is important because it is involved in DNA synthesis, cell growth, embryonic development, and function of the nervous system. Per definition of a vitamin it is not synthesized *de novo* in the body, and thus, must be obtained through diet either in the naturally occurring reduced forms or by supplementation and fortification in the form of folic acid (synthetic form). The recommended dietary allowances of folate vary with age and health status. Folate occurs abundantly in green vegetables, meat products, citrus fruits, and whole grains. However, it is thermally liable; thus harvesting, storage, and food preparation, can decrease naturally occurring folate levels in food. In 1996, after the recognition that low folate levels during pregnancy could result in neural tube defects (NTDs) and supplementation with folic acid prevented NTDs (Butterworth and Bendich 1996), the Food and Drug Administration mandated the fortification of folate in the form of folic acid in foods such as: cereal, flour, corn meal, bread, pasta, rice, and other grain products (Food Standards 1996). Today, there are also a vast number of over-the-counter supplements available that contain folic acid. Additionally, a small amount of folate is derived from

the colonic folate producing bacterial flora (Keagy and Oace 1984). Both synthetic and naturally occurring folate are commonly referred to as simply "folate".

Folate Derivatives

Folate exists in a number of different oxidative states. All forms of folate have the same three structural components (Figure 1). There is a pteridine moiety linked through a methylene bridge to a *p*-amino benzoate group, which is attached via a peptide linkage to glutamate (GLU). Dietary folate is usually in the reduced form, and contains a conjugated side chain of glutamate molecules ranging from one to seven residues (Rampersaud et al. 2003). These polyglutamate folates must be hydrolyzed via a zinc-dependent pteroylglutamate hydrolase to the monoglutamate form before being ingested into the intestinal tract. Synthetic folate (folic acid) occurs in the monoglutamate form, thus making it readily bioavailable since it does not require intestinal enzymatic cleavage prior to absorption (Rampersaud et al. 2003).

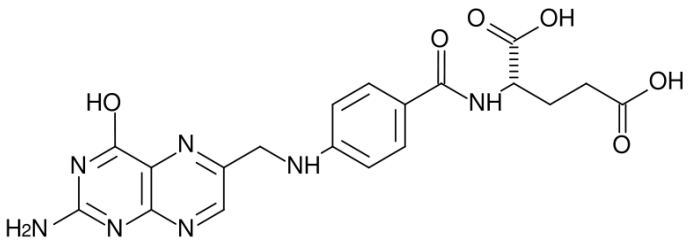


Figure 1. Chemical structure of folate.

Folate Transport

Carrier-mediated transport systems are used for the uptake of monoglutamate folate derivatives. There are three types of transport systems involved in the transport of folate: proton-couple folate transporter (PCFT), reduced folate carrier (RFC), and a small

family of folate receptors (FRs) encoded by FRα, FRβ, FRγ, genes. PCFT is responsible for transport to the upper small intestine (Stockstad et al. 1977). RFC is found ubiquitously in the body and is responsible for transport to the colon, renal tubes, placenta, and blood-brain barrier (BBB); it has highest affinity for reduced folates (Stockstad et al. 1977). FRα and FRβ are glycosylphosphatidylinositol anchored receptors which function through receptor-mediated endocytosis (Butterworth et al 1974). They have the highest affinity for 5-methyltetrahydrofolate (5-MTHF) and a lesser attraction for other reduced monoglutamate derivatives (Antony 1992; Westenhof et al. 1995).

Monoglutamate folate is transported into the intestinal cells by RFC. Once in the cell, the pteridine can be reduced at carbons 5, 6, 7, and/or 8, resulting in active forms of folate that act as cofactors in numerous biochemical reactions. These reduced forms can carry one-carbon units either as methyl, methylene, or formyl groups attached to the N-5 and/or N-10 position of tetrahydrofolate (THF). Once in the active form, it exits the cell to enter the folate pool in the blood stream. When the monoglutamate form is taken up into cells the glutamate chain can be extended in reactions catalyzed by folylpolyglutamate synthetase assuring that the coenzyme remains in the cell for storage. 5-MTHF is the physiologically active form that is transported across cell membranes from the circulation, including the BBB, allowing it to enter the CNS. The folate derivative binds to FRα located on the basal side of the choroid plexus epithelial cells. After endocytosis it is removed from the cell into the cerebral spinal fluid (CSF) via RFC located on the apical membrane. Compartmented 5-MTHF is then taken up in neural tissue mediated by RFC. The active uptake at the BBB and accumulation of folate in

CSF results in a concentration that is two to three times higher than in plasma (Spector et al. 1975; Reynolds et al. 1972; Gallagher 1983).

Folate Metabolism and Related Pathways

Folate Cycle of One-Carbon Metabolism

The involvement of folate in one-carbon metabolism is depicted in figure 2. Monoglutamate folate is reduced first to dihydrofolate (DHF) and, then onto THF. Both reactions are catalyzed by the enzyme dihydrofolate reductase (DHFR). From here 5,10-methylene-THF is formed from the reaction of THF with serine as the one-carbon unit donor. The reaction is catalyzed by serine hydroxymethyltransferase (SHMT). The 5,10-methylene-THF is the derivative responsible for nucleotide synthesis. Specifically, deoxyuridine monophosphate (dUMP) is converted into deoxythymidine monophosphate (dTMP) by transfer of one carbon from 5,10-methylene-THF to dUMP via thymidylate synthetase (TS), resulting in the formation of dTMP, which is incorporated into nucleotides (Carreras and Santi 1995). THF is also responsible for the generation on 10-formyl-THF, which is involved in purine synthesis (Brodsky et al. 1997). Subsequently, 5,10-methylene-THF is further reduced via methylene-THF reductase (MTHFR) to 5-MTHF, the primary form found circulating in body. Next methionine synthase (MTR) facilitates the methyl group transfer from 5-MTHF to cobalamin to form methylcobalamin. MTR transfers the methyl groups from methylcobalamin to homocysteine (Hcy) generating methionine (MET), THF, and cobalamin (Drennan et al. 1994). The liver and kidney can also use betaine (BET) as a methyl donor to regenerate methionine via betaine homocysteine methyltransferase (BHMT) (Sunden et al. 1997).

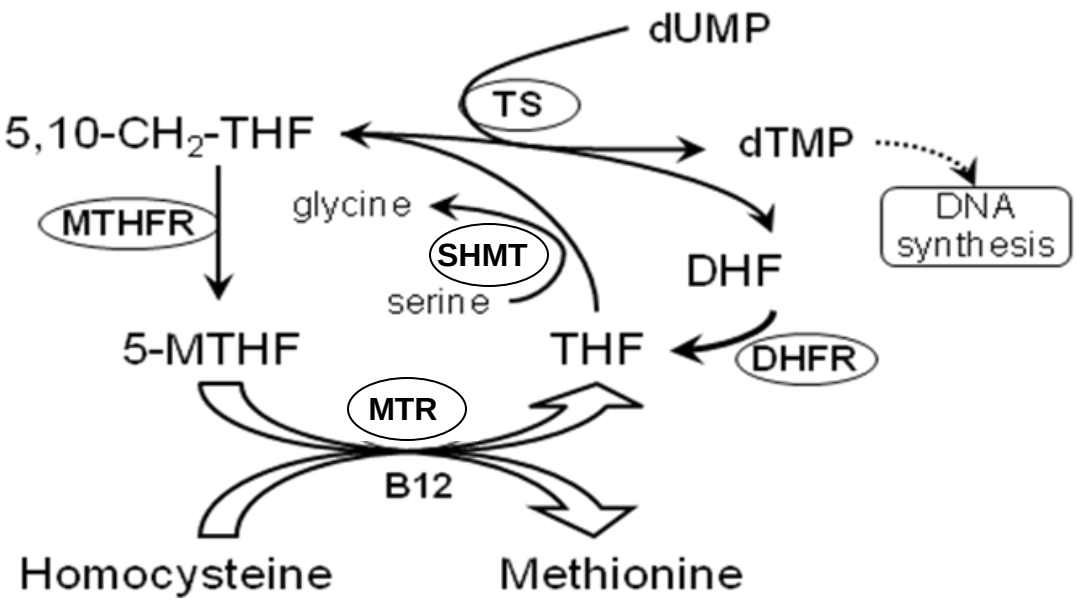


Figure 2. Folate pathway of one-carbon metabolism. Adapted from Bottiglieri and Reynolds 2010.

Methylation Cycle

The intricate system in which a methyl group is transferred between various moieties involved in the folate and methylation cycles is depicted in figure 3. Upon formation methionine has two fates; it can either be incorporated into polypeptide chains or consumed to generate S-adenosylmethionie (SAM). *De novo* synthesis of methionine is important because dietary intake is not sufficient to sustain levels of SAM required by the body. SAM is formed by the adenosylation of methionine using the enzyme, methionine adenosyltransferase (MAT). SAM is the major methyl donor throughout the body. It is involved in numerous methyltransferase reactions, including methylation of DNA, proteins, neurotransmitters and phospholipids (Cheng and Blumenthal 1999). After donating its methyl group to these various molecules S-adenosylhomocysteine (SAH) is formed. Under normal physiological conditions SAH is rapidly hydrolyzed to

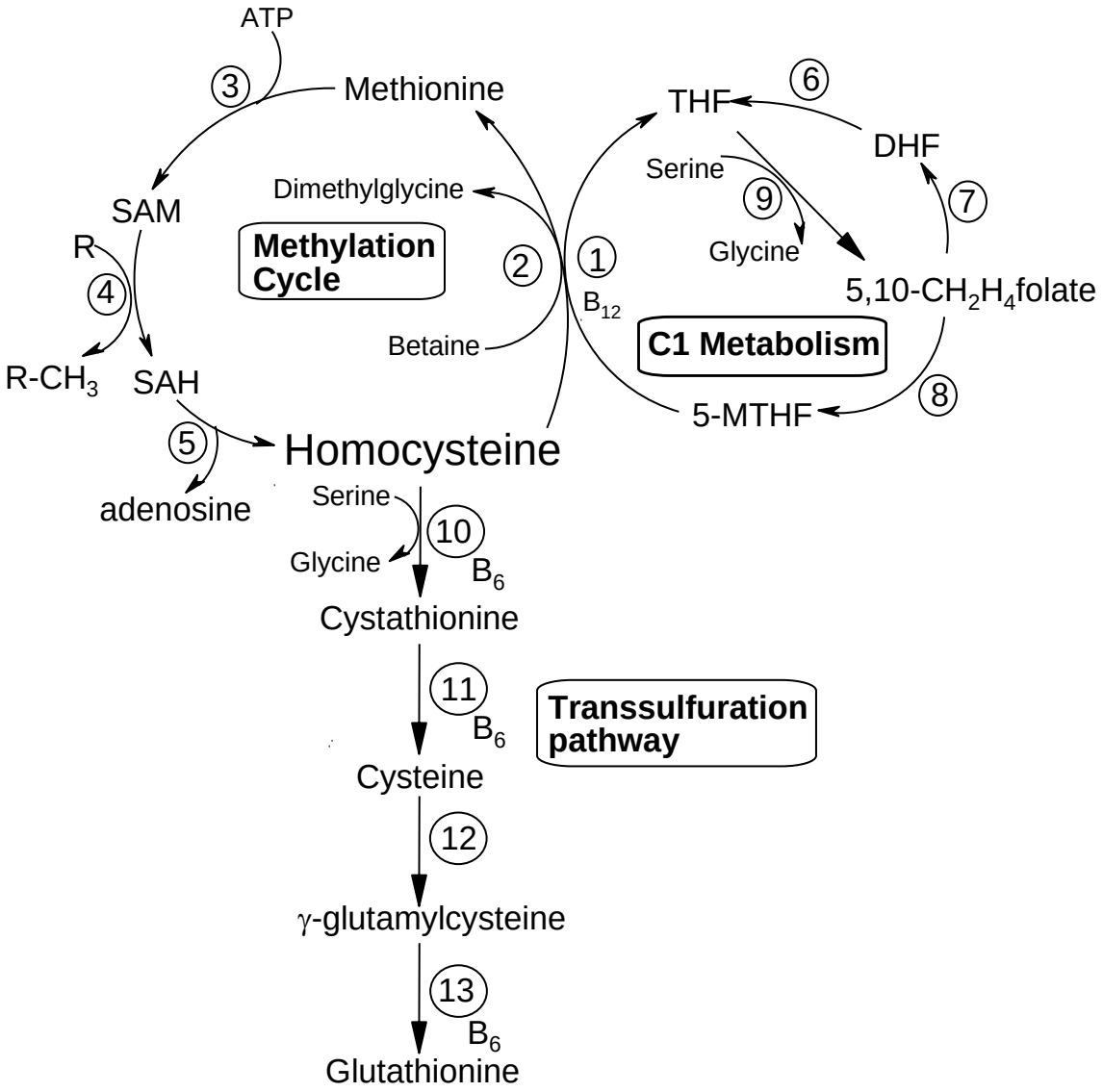


Figure 3. Relationship between the metabolic pathways of one-carbon metabolism, methylation, and transsulfuration. Key enzymes: 1, methionine synthase; 2, betaine-homocysteine methyltransferase; 3, methionine adenosyltransferase; 4, R-methyltransferases; 5, S-adenosylhomocysteine hydrolase; 6, dihydrofolate reductase; 7, thymidylate synthase; 8, 5,10-methylentetrahydrofolate reductase; 9, serine hydroxy-methyltransferase; 10, cystathionine β-synthase; 11, cystathionine γ-lyase; 12, γ-glutamylcysteine synthase; 13, glutathionine synthase.

Hcy, a sulfur containing non-protein forming amino acid, along with adenosine (ADE), as a by-product via the enzymatic activity of S-adenosylhomocysteine hydrolase (SAHase). This reaction is reversible; under conditions of high Hcy concentrations SAH is favored via SAHase. Hcy, at this point, has three fates, mainly determined by cystathionine β-synthase (CBS), and MTR activity. It can be converted to methionine via MTR or BHMT, as previously mentioned. Hcy can also enter the transsulfuration pathway to form cystathionine (CYSTA) and then cysteine (CYS) in a reaction catalyzed by the vitamin B_6 dependent enzyme CBS. Lastly, if either CBS or MTR activity is restricted, then Hcy can be converted back to SAH by SAHase.

This pathway is highly regulated with both feed-back and feed-forward activation and inhibitory mechanisms. For instance SAM is an inhibitor of MTHFR while an activator of CBS, hence when SAM levels are elevated the transsulfuration pathway is upregulated, and the folate cycle downregulated, thus blocking the generation of additional SAM (Finkelstein 1998; Janosik et al. 2001). Also when MTR activity is decreased there is an increase in BHMT activity (Barak et al. 1984). In addition, BHMT activity is subjected to feed back inhibition by methionine and even more so by dimethylglycine (Finkelstein et al. 1972; Allen et al. 1993). SAH is an inhibitor of many methyltransferase reactions thus it too is a regulator of the pathway (Kerr 1972). These mechanisms of regulation are extremely important in controlling the flux of the pathways.

Transsulfuration Pathway

Methionine and cysteine are also responsible for the regulation of one-carbon metabolism. If methionine levels are low, then synthesis is favored from Hcy. However,

if methionine and cysteine are in an adequate supply, SAM beings to accumulate, which activates CBS and the transsulfuration pathway (Finkelstein et al. 1975). The first step in the transsulfuration pathway is the formation of cystathionine and glycine from the condensation of Hcy with serine via CBS. Subsequently, cystathionine is cleaved by cystathionine-γ-lyase (CSL) forming cysteine as the major product; however, studies have shown that CSL is not present in brain and heart tissue (Ishii et al. 2004). During two adenosine triphosphate dependent steps cysteine is metabolized to glutathione (GSH), the major antioxidant molecule in the cell. This is the primary function of the transsulfuration pathway.

Folate Mechanisms of Toxicity in the Central Nervous System

There are a variety of disorders of the CNS associated with folate deficiency. These include but are not limited to: depression, mental disturbances, stroke, epilepsy, NTDs, multiple sclerosis, Alzheimer's disease (AD) and age related cognitive decline (Czeizel and Dudas 1992; Hibbard et al. 1965; Morris et al. 2008; Bottiglieri 2005; Bottiglieri et al. 2000; Nelson et al. 2009; Brown et al. 2007). The exact mechanism(s) of the pathogenesis caused by folate deficiency in many disorders including cognitive decline remains unclear, although several mechanisms have been proposed. Folate deficiency, whether induced by dietary, genetic, or drug interactions, results in low CNS 5-MTHF levels. The hypothesized mechanisms in which inadequate folate levels cause toxicity to the CNS is outlined in figure 4.

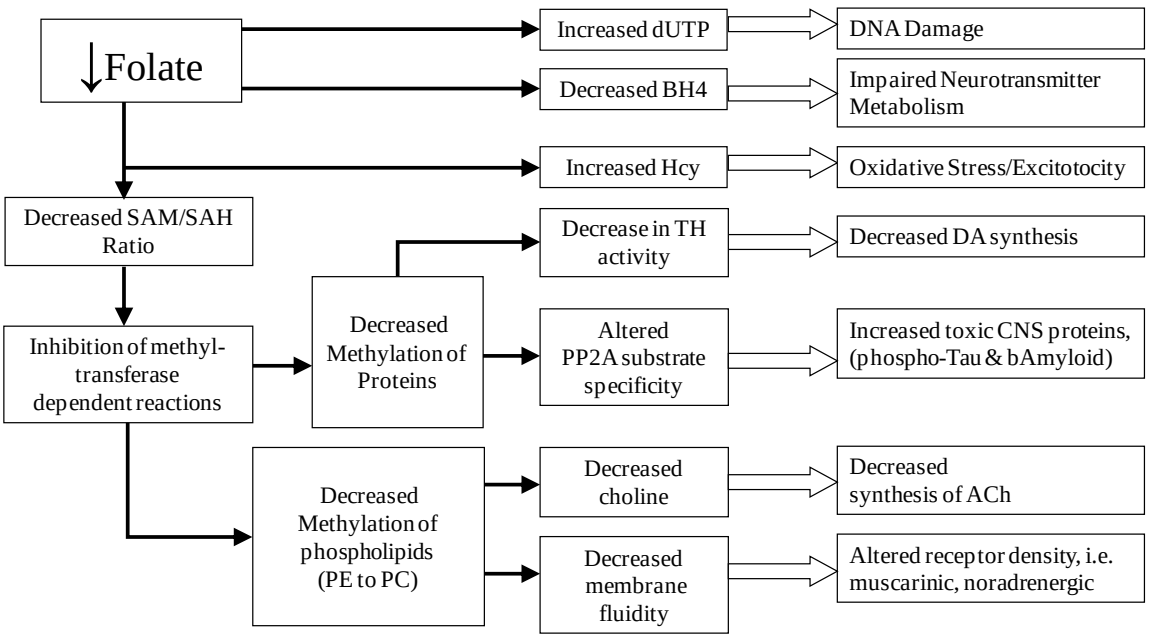


Figure 4. Folate deficiency: mechanisms for toxicity.

Increased dUTP

The nucleotide dTMP is required for DNA synthesis. Production of dTMP is dependent upon 5,10-methylene-THF. 5,10-methylene-THF transfers its methylene group to the 5-carbon of dUMP to form dTMP and DHF. Thymidylate synthetase (TS) catalyzes this reaction, which if blocked results in accumulation of dUMP and dUTP along with a concomitant decrease in dTMP. During times of increased dUTP levels, misincorporation of uracil instead of thymidine occurs, resulting in A:U mismatched pairs in the DNA strand, leading to genomic instability and apoptosis (Beetstra et al. 2005; Kruman et al. 2004). Under normal conditions, this phenomenon does occur; however, the DNA is repaired by a base excision-repair (BER) pathway involving uracil-DNA *N*-glycosylate (UNG). Thus, when levels of 5,10-mthylene-THF are low, levels of dUTP are increased, resulting in an increase of A:U mismatches. The CNS in particular is more vulnerable to DNA damaging agents (Morris et al. 1996). It is also thought that

the aging brain is more prone to DNA damage because in murine models it was found that the BER pathway drops significantly with increasing age (Gredilla et al. 2010). Furthermore, support for this mechanism of toxicity can be found in a study that reported mice lacking UNG performed poorly on cognitive testing when treated with a folate deplete diet (Kronenberg et al. 2008).

Decreased BH_4

Tetrahydrobiopterin (BH_4) is a cofactor required for the synthesis of the monoamine neurotransmitters, dopamine (DA), norepinephrine (NE), and serotonin (5-HT) which have all been implicated in etiology of a wide variety of psychiatric disorders (Kaufman 1981). There are two routes by which BH_4 is formed: a *de novo* pathway and a salvage pathway (Figure 5). Briefly, in *de novo* synthesis, guanidine triphosphate (GTP) is converted to BH_4 through several reactions involving the action of three enzymes: GTP cyclohydrolase (GTPCH), 6-pyruvoltetrahydrobiopterin synthease (PTPS), and sepiaterin reductase (SR). The salvage pathway of BH_4 involves the conversion of quinonoid dihydrobiopterin (qBH_2) by dihydropteridine reductase (DHPR). There is evidence that MTHFR and DHFR, two enzymes involved in folate metabolism, might also play a role in the salvage pathway due to the fact that both folate and BH_4 contain a pterin moiety (Kaufman 1981; Kaufman 1991). This metabolite is the rate-limiting cofactor for both tryptophan hydroxylase (TPH) and tyrosine hydroxylase (TH).

Tryptophan (TRP) is hydrolyzed to 5-hydroxytryptophan (5-HTP) via TPH accompanied by BH_4. In the second step, 5-HTP is decarboxylated by the action of amino acid decarboxylase (DDC) in the presence of B_6 to yield serotonin. After inducing its action on receptors most is recycled by serotonergic neurons, however some is

catabolized by monoamine oxidase (MAO) and aldehyde dehydrogenase to produce 5-hydroxyindoleacetic acid (5-HIAA).

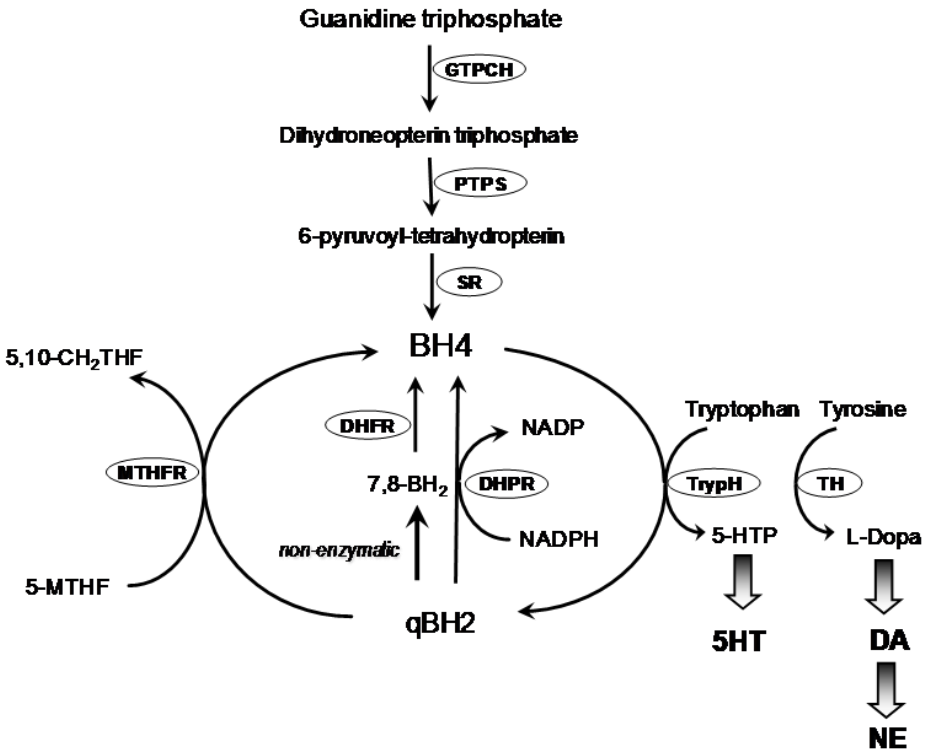


Figure 5. Relationship between folate and monoamine neurotransmitter synthesis. Adapted from Bottiglieri and Reynolds 2010.

For the synthesis of dopamine, tyrosine (TYR) is hydrolyzed to levodopa (L-DOPA) via TH using BH_4 as the cofactor. L-DOPA is decarboxylated to form dopamine by DOPA decarboxylase. From this point dopamine has many fates. It can be used in the synthesis of the monoamine neurotransmitters norepinephrine and epinephrine or it can be degraded by two main pathways. In most areas of the brain, dopamine is inactivated by reuptake via the dopamine transporter (DAT) then broken down by MAO to 3,4-dihydroxyphenylacetic acid (DOPAC), which is further degraded to 3-methoxy-4-hydroxy-phenylacetic acid (HVA). In other regions of the brain where DAT is scarce

reuptake of dopamine is accomplished by norepinephrine transporter then catabolized to 3-methoxytyramine (3-MT) via catechol-O-methyltransferase (COMT). Dopamine can also be repackaged into vesicles for reuse.

Under circumstances of low folate, BH_4 concentrations may be altered, resulting in decreased monoamine neurotransmitter levels. This proposed mechanism of toxicity is supported by several studies. For instance, children with in-born errors of folate metabolism present metabolically with reduced levels of dopamine and serotonin (Surtees et al. 1994). Patients with MTHFR deficiencies, folate deficient mice, and low folate-depressed patients all showed decreased levels of BH_4, dopamine, and serotonin, consistent with a role for folate in BH_4 synthesis and monoamine neurotransmitter metabolism (Kaufman et al. 1991; Gospe et al. 1995; Bottiglieri et al. 2000). More support is drawn from a study that supplemented rats with 5-MTHF and found increased levels of BH_4 (Hamon et al. 1986).

Increased Homocysteine

Homocysteine exists in multiple forms; a free thiol, disulfide, mixed disulfide and protein bound thiol groups are present (Jacobsen 1998). Approximately 75% of plasma Hcy is protein bound, primarily to albumin, the non-protein bound forms (Hcy-Hcy, Hcy-CYS, and Hcy) make up the remaining 25% (Refsum et al. 1985; Mudd et al. 2000). Free Hcy rapidly complexes with other sulphur compounds through disulphide bonds, thereby, what is typically measured is total Hcy (tHcy) after reduction to the free form. Hcy is efficiently transported out of cells (Svardal et al. 1986); hence the concentration in plasma reflects a balance between intracellular Hcy production and utilization (Bottiglieri 2005).

Plasma tHcy is a sensitive marker of folate and vitamin B_{12} status. When folate levels are diminished, the normal re-methylation of Hcy to methionine is limited, therefore resulting in an accumulation of Hcy, referred to as hyperhomocysteinemia (HHcy). The increase of Hcy can be exacerbated in the presence of enzyme defects of MTR, CBS, or MTHFR genes. Thus, low folate, in combination with a mutation resulting in lower enzyme activity, increases the risk of developing HHcy. Increased tHcy levels have been reported in children with severe MTHFR deficiencies, depressed and demented patients with low folate levels and in murine models fed treatment diets with inadequate folate content (Surtees et al. 1994; Bottiglieri et al. 1990; Fuso et al. 2008; Troen and Rosenberg 2005). HHcy has also been shown to be associated with cognitive decline. There is a considerable amount of literature that shows increased Hcy concentration is toxic to both vascular endothelial cells and neuronal cell. The mechanisms involved are outlined in the following sections.

Endothelial dysfunction and disease. There is extensive literature indicating that HHcy is detrimental to vascular endothelium and is associated with stroke, heart disease, cerebrovascular disease, and vascular dementia (McCully and Wilson 1975; Giles et al. 1998; Robinson et al. 1998; Voutilainen et al. 2001). HHcy induces vascular endothelium dysfunction by reducing the bioavailability of nitric oxide (NO), a gaseous compound derived from arginine (ARG). It is responsible for regulation of cerebral blood flow, and inhibits fibrosis, and proliferation of smooth muscle cells in the arterial wall (Hassan et al. 2004; Iadecola et al. 1994; Nasreen et al. 2002). In addition, NO facilitates vascular repair by endothelial progenitor cells (Iadecola et al. 1994). The exact mechanism of how HHcy reduces NO remains unclear; however there is evidence to

propose that the generation of superoxides during HHcy may result from uncoupling of endothelial nitric oxide synthase (eNOS) the enzyme used for the synthesis of NO from arginine (Boger et al. 2003).

In addition, HHcy has been shown to be correlated with levels of asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) (Guldiken et al. 2007; Dayal and Lentz 2005; Stuhlinger et al. 2003). ADMA is a competitive inhibitor of eNOS and thereby may reduce NO concentrations (Boger et al. 1998; Boger et al. 2003). The ensuing uncoupling of eNOS and reduction in NO leads to endothelial dysfunction. Previous studies have shown that ADMA is actually more potent in inhibiting neuronal NOS than eNOS *in vitro* (Kielstein et al. 2007). Indeed, higher levels of ADMA have also been associated with defects in cerebral circulation and cerebrovascular diseases (Faraci et al. 1995; Kielstein et al. 2006; Pluta 2005; Khan et al. 2007).

ADMA and SDMA are formed from the post translational methylation of arginine residues in proteins via the action of protein arginine methyltransferase (PRMT) with SAM participating as the methyl donor in this reaction. These methylation metabolites are released from proteins during proteolysis and exported into the cytoplasm and eventually into circulation. Both are eliminated via renal clearance. However, only ADMA is metabolized to citrulline via dimethylarginine dimethylaminohydrolase (DDAH) (Figure 6). Studies have shown that Hcy can inhibit DDAH and that this leads to elevation of plasma ADMA (Tyagi 2005 et al.; Stuhlinger and Stanger 2005). Mice over-expressing DDAH have reduced plasma ADMA levels, and increased vascular responses of carotid arteries. Addition of exogenous ADMA impaired the response in

both DDAH *tg*-mice and control mice; however, DDAH *tg*-mice still had greater relaxation of the carotid arteries (Rodionov et al. 2010). Another group reported hypermethylation in the promoter cytosine-phosphate-guanidine (CpG) island of DDAH in human umbilical vein endothelial cells treated with 100 µM Hcy, which was associated with decreased transcription of DDAH mRNA (Zhang et al. 2007).

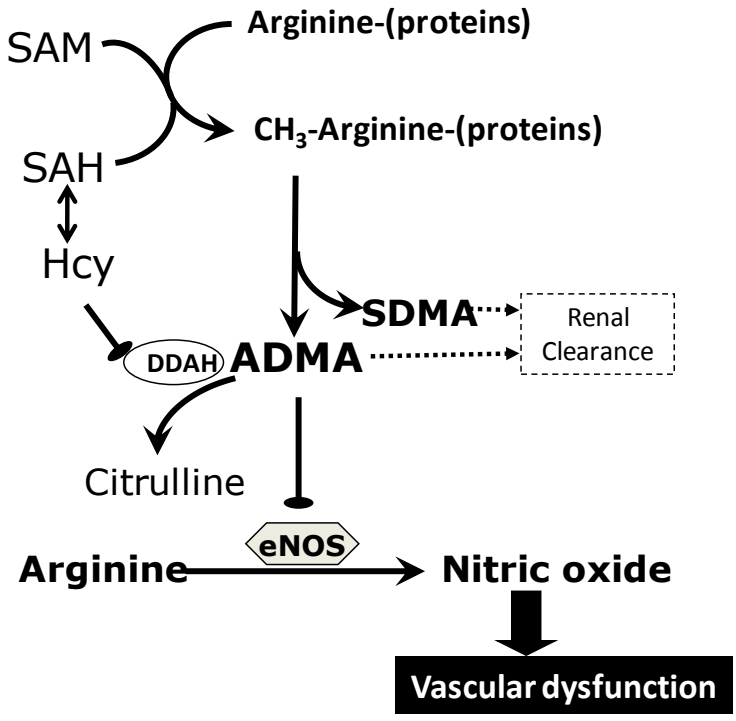


Figure 6. ADMA synthesis.

Excitotoxicity. It has been speculated that Hcy can spontaneously oxidize to homocysteine sulfinic acid (HCSA) and homocysteic acid (HCA). Enzymatic activity for these reactions has not been confirmed. Formation of both HCSA and HCA has been reported in the brain using labeling studies (Do et al. 2004; Cuenod et al. 1993). These metabolites, commonly referred to as excitatory sulfur amino acids (ESAA). are agonists for N-methyl-D-aspartate (NMDA) receptors, which are a subgroup of excitatory

glutamate receptors (Lipton et al. 1997). Upon binding to the NMDA receptors, there is an unwarranted activation leading to an influx of calcium and eventual cell death. Therefore, these two ESAAs have the potential for extensive neuronal damage. Children with Kawasaki disease have elevated HCA plasma levels and folic acid supplementation to these patients significantly reduced the levels of HCA from 7.79 +/- 1.79 µM to 4.56 +/- 1.14 µM (Chu et al. 2007).

Oxidative stress. Oxidative stress induced by free radicals has been associated with many CNS disorders. It has been postulated that HHcy causes an increase in reactive oxygen species (ROS), although the exact mechanism is unclear. One pathway of oxidative stress induced by ROS is depicted in figure 7. The ROS, superoxide radical is converted to hydrogen peroxide (H_2O_2) by the action of superoxide dismutase (SOD), which is then removed by catalase or glutathione peroxidase (GPx). However, catalase is not present in brain; thus, GPx is the major mechanism responsible for its removal via coupling with the antioxidant GSH. Oxidized glutathione (GSSG), the product of this reaction is recycled back to the active form by glutathione reductase (GRed). The majority of the intracellular pool of GSH is derived via the transsulfuration pathway from cysteine as the precursor (Mosharov et al. 2000). Diminished levels of GSH result in H_2O_2 breaking down into hydroxyl radicals (OH), which are damaging to the cell due to their oxidizing capabilities. Folate deficiency in SH-SY5Y neuroblastoma and cortical cells leads to an increase in ROS (Ho et al. 2003). Ovrebo and colleagues demonstrated that Hcy was negatively correlated with both CYS and GSH levels in rat brain tissues (Ovrebo and Svardal 2000).

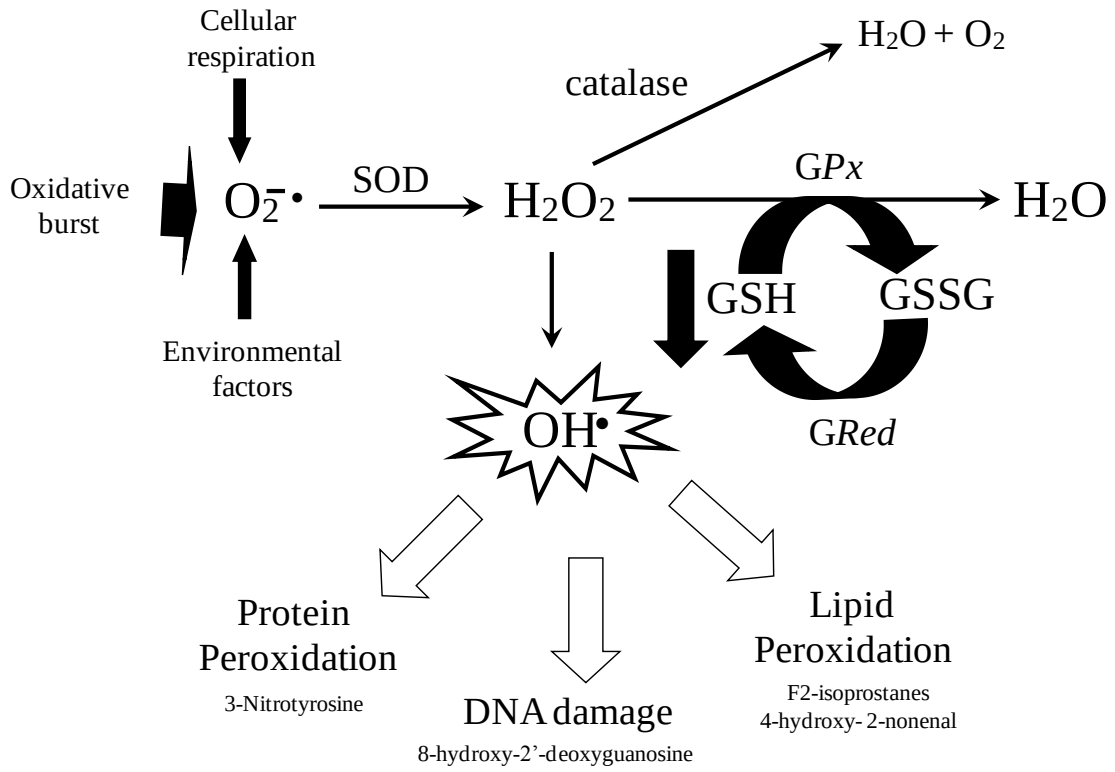


Figure 7. The role of GSH in oxidative stress.

A recent study also showed a positive correlation in AD patients with low folate status between plasma Hcy and 4-hydroxy-2-nonenal levels, a marker of lipid peroxidation (Selley et al. 2002).

Decreased SAM/SAH Ratio

Numerous methyltransferase enzymes are responsible for catalyzing the transfer of a methyl group to a variety of different substrates, such as DNA, proteins, and phospholipids (Chiang et al. 1996; Shelhub et al. 1999). SAM is the universal methyl donor in all of these methyltransferase reactions and produces SAH as a by-product. Usually SAH is rapidly converted to Hcy. However, during periods of inadequate folate, intracellular Hcy levels increase, and can then be metabolized in a reverse SAH hydrolase

reaction to SAH (Dayal et al. 2001). SAH accumulation can be detrimental because it has been shown to be a competitive inhibitor of methylation reactions (Cantoni 1985). The SAM/SAH ratio is often used as an indicator of methylation status, with a low ratio representing a deficiency. Aging and/or other diseases including a variety of cancers, AD and Parkinson's disease (PD) have been shown to be associated with hypomethylation (Bottiglieri et al. 1990; Morrison et al. 1996; Socha et al. 2009; Wilson et al. 1987; Richardson 2003; Obeid et al. 2009). It has been reported that in PD patients there is an association between cognitive function and the SAM/SAH ratio, suggesting that there is a role for methylation in this neurodegenerative disease (Obeid et al. 2009).

DNA. Methylation of DNA using SAM as the substrate occurs on the 5' position of the cytosine pyrimidine ring or the 6'-N of the adenine purine ring via the action of DNA methyltransferase. Folate metabolism supports the synthesis of SAM and thus appears to be a regulator of DNA methylation. Methylation typically occurs within CpG rich sites along the genome with approximately 60-90% being methylated. DNA methylation is important for: suppression of viral genes and other deleterious elements incorporated over time, altering gene expression patterns in cells, and controlling gene regulation. It is also known that DNA extracted from mammalian brain has the highest concentration of 5-methylcytosine compared to other tissues, thus, methylation plays a central role in CNS function, with an inverse relationship between CpG methylation and transcriptional activity (Vanyushin et al. 1973).

Presenilin 1 (PS1) gene expression is one important example that has been tested in relation to methylation. PS1 is a regulator of γ-secretase, one of the enzymes that mediate Aβ formation in AD patients by cleavage of the amyloid precursor protein

(APP). Under normal conditions the promoter regions of these enzymes are hypermethylated, thereby suppressing their action. During conditions of hypomethylation, they become active increasing Aβ levels. In one study, neuroblastomas deprived of folate and B_{12} had a reduction in SAM, while inducing PS1 and β-Secretase (BACE) promoter hypomethylation, along with an increase in protein expression in both enzymes; this resulted in an increase of Aβ production (Fuso et al. 2005). These findings were also replicated in a transgenic mouse model for AD (Fuso et al. 2008).

Proteins. There are a large number of post translational modifications that can occur to proteins, all of which serve to expand the repertoire of biochemical actions that a protein can perform. Methylation of proteins using SAM as the substrate is a common post translational modification that occurs. Arginine, lysine, and leucine are the amino acid residues classically methylated in a protein sequence on the nitrogen atom, with both able to accept more than a single methyl group. Protein phosphatase 2A (PP2A) is a heterotrimeric enzyme belonging to a class of serine and threonine phosphatases. One of the primary functions of PP2A is dephosphorylation phospho-Tau (p-Tau). The accumulation of p-Tau is toxic to neurons and known to play a role in the pathogenesis of AD. PP2A consists of 3 subunits: A, C, and Bα. The A and C subunits readily combine. However, methylation at the leucine-309 residue on the catalytic C subunit must occur in order for the third Bα subunit to combine with the AC units. In this trimeric form PP2A has an increased binding affinity for the dephosphorylation of Tau protein (Sontag et al. 1996; Nunbhakdi-Craig et al. 2007). This mechanism of toxicity is supported by a recent study in which mice were fed either control, low folate, or folate deficient diet for 2 months. After treatment, mice fed a folate deficient diet had a reduced SAM/SAH ratio

in brain tissues, accompanied by decreased methylation of PP2A, specifically at the C subunit, and increased levels of p-Tau (Sontag et al. 2008).

Another post-translation modification of a protein of significance is the methylation of myelin basic protein (MBP), which is essential for the proper functioning of the CNS. Hypomethylation of MBP at arganine-107 results in demyelination. A study of patients who had demyelination, of which 10.7% were folate deficient, showed that 67% of the folate deficient patients improved after supplementation with 15 mg/day of folic acid (Yukawa et al. 2001).

Phospholipids. There are two phospholipid methyltransferase enzymes (PEMTs) which are distributed asymmetrically in membranes of all nucleated mammalian cells. They are responsible for the conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC), that involve the transfer of three methyl groups derived from SAM. Upon sequential methylation, PE is translocated from the inside to the outside of the membrane causing an increase in membrane fluidity, resulting in movement of proteins (Crews 1982). Studies in PEMT-KO mice suggest that PC synthesis is quantitatively the most significant SAM dependent reaction and therefore the most dominant source of Hcy production (Stead et al. 2006). PC levels have been shown to be decreased in CNS tissue from mice fed a folate deficient diet for 10 weeks. These mice also had a decline in spatial memory (Troen et al. 2008).

Choline (CHO) is derived from the breakdown of PC. Choline is the substrate for the neurotransmitter, acetylcholine (Ach), and the methyl donor, betaine. Acetylcholine plays an important role in learning and memory, and is altered in many diseases involved in age-related cognitive decline (Freeman and Gibson 1988; Drachman et al. 1973).

Choline is oxidized by choline oxidase to form betaine aldehyde. In a sequential step betaine is synthesized through the action of betaine aldehyde dehydrogenase. Not only is betaine involved in one-carbon metabolism but it also serves as an osmoregulator. Thus, tissue concentrations are much higher than plasma concentrations (Slow et al. 2009).

Causes of Folate Deficiency and Populations at Risk

Folate deficiency is defined as having plasma folate levels below 5 nM or red blood cell (RBC) levels below 305 nM, although these numbers do vary with age and health status (Rampersaud et al. 2003). Pfeiffer and colleagues compiled folate data from the National Health and Nutrition Examination Survey (NHANES) in the US before (1988-1994) and after folic acid fortification (1999-2000). They found the prevalence of folate deficiency declined from 16% to 0.5% over the study period (1988-2000) (Pfeiffer et al. 2005). More recently Frankenburg published data stating that the occurrence of low folate status had decreased from about 22% to 2% since fortification (Frankenburg 2007). Nonetheless, even though folate deficiency is on the decline, there are certain populations that may be at risk for this important nutritional deficiency. For example, folate deficiency can occur when an increased need for folate (pregnancy and genetic factors), is not matched by an increase in consumption, when dietary folate intake does not meet recommended needs, and when folate loss increases due to medical conditions (alcoholism, liver or kidney disease, and certain anemias). Also, certain medications (anticonvulsants, methotrexate, sulfasalazine, and barbiturates) can interfere with folate metabolism or absorption, resulting in a state of folate insufficiency.

There are at least three major enzymes involved in the folate and methylation pathways with known mutations resulting in altered enzyme activity: MTHFR, MTR, and

CBS. Numerous single nucleotide polymorphisms (SNPs) have been reported in each of these genes and all have different frequencies and characteristic traits. Table 1 gives an overview of the frequency of the rare alleles of the most common mutations. The table also demonstrates the tremendous influence demographics have on the prevalence of the SNPs.

Table 1. Human polymorphisms affecting folate and methylation metabolism. Adapted from Fredriksen et al. 2007 and Lissowska et al. 2007.

Gene	DNA Change	Protein Change	Frequency of rare allele	Population Frequencies (-/-)	
MTHFR	677 C>T	ALA-VAL	28%	US	15%
				Korea	16%
				Australia	11%
				Poland	10%
MTFHR	1298 A>C	GLU-ALA	34%	Poland	11%
MTR	2756 A>G	ASP-GLY	19%	US	1-5%
				Korea	2-3%
				Europeans	3%
				Poland	4%
MTRR	66 A>G	LEU-MET	60%	US Hispanics	50%
				Hawaii	8-10%
				Africans	42%
				Poland	18%
CBS	844_845INS68		9%	All populations	rare
CBS	699 C>T	TRY-TRY	33%	Poland	12%

A deficiency of MTHFR is the most common of all inherited disorders of folate metabolism with over forty mutations being reported to date (Cooper 1987; Rosenblatt 1995; Sibani et al. 2000). Brotto and Yang reported that 20% of the world's population has polymorphisms in the MTHFR gene that decrease its activity (Brotto and Yang 2000). One common MTHFR polymorphism which results in a mild deficiency is the MTHFR C677T mutation. In the US, the MTHFR C677T allele mutation frequency is

15% and the prevalence is highest among Hispanics, followed by Caucasians, and lastly African Americans, suggesting that a large portion of the US population may be at risk for altered folate metabolism. The MTHFR C677T mutation causes heterozygous carriers to have approximately 60-70% enzyme activity whereas homozygous carriers have only 30% enzyme activity. Homozygotes also have an 18% decrease in RBC 5-MTHF levels, and increased levels of 10-formyl-THF, 5,10-methylene-THF, in addition to tHcy elevations (Rozen 1996.). Some studies have linked the MTHFR C677T genotype to depression, dementia, cardiovascular disease, premature vascular disease, and schizophrenia (Gilbody et al. 2007; Lewis et al. 2006; Frosst et al. 1995; Roffman et al. 2008; Gorgone et al. 2009; Kluijtmans et al. 1996). There are also a limited number of homozygous carriers with less than 20% enzyme activity (less than 50 cases worldwide). Individuals with severe MTHFR deficiency typically exhibit developmental delay, motor and gait dysfunction, seizures, and neurological impairment (Rosenblatt 1995; Sibani et al. 2000). Another common MTHFR polymorphism that also leads to a decrease in enzymatic activity is the A1298C mutation. This homozygous population has approximately 60-70% MTHFR activity, resulting in decreases in 5-MTHF levels; however, no increase in Hcy was reported (Weisberg et al. 1998; Freidman et al. 1999). Hence, hereditary in-born errors of folate metabolism limit the one-carbon groups in circulation, thus magnifying the effect of insufficient dietary folate.

Carriers of the SNPs are not the only group of people with an increased risk for folate deficiency. Pregnant women are also at a high risk due to the elevated metabolic demands for folate during times of the rapid neonatal growth, since folate is tightly involved with DNA synthesis. Populations that are nutritionally deficient due to poverty

or disease are also in a higher quartile for being in a state of negative folate balance. For instance, a 1997 review of the nutritional status of people suffering from alcoholism had 50% lower folate status than controls due to alcohol interfering with folate absorption, increased kidney excretion, and a typically poor diet (Gloria et al. 1997; Cravo et al. 1996). Many other diseases also interfere with folate metabolism including, but not limited to: kidney dialysis, liver disease, achlorhydria, and hypochlorhydria.

It is known that there is an age-related decline in folate status after 70 years of age with an increase in Hcy levels (Bottiglieri et al. 2000; Serot et al. 2005; Rosenberg 2001; Herrmann et al. 2007) (Figure 8). Researchers speculate the reason for this age-related decline in folate is due largely in part to increased prevalence of intestinal malabsorption status, and, to a lesser extent insufficient dietary folate intake. It is estimated by various that that atrophic gastritis ranges anywhere from 20-50% in the elderly population, which

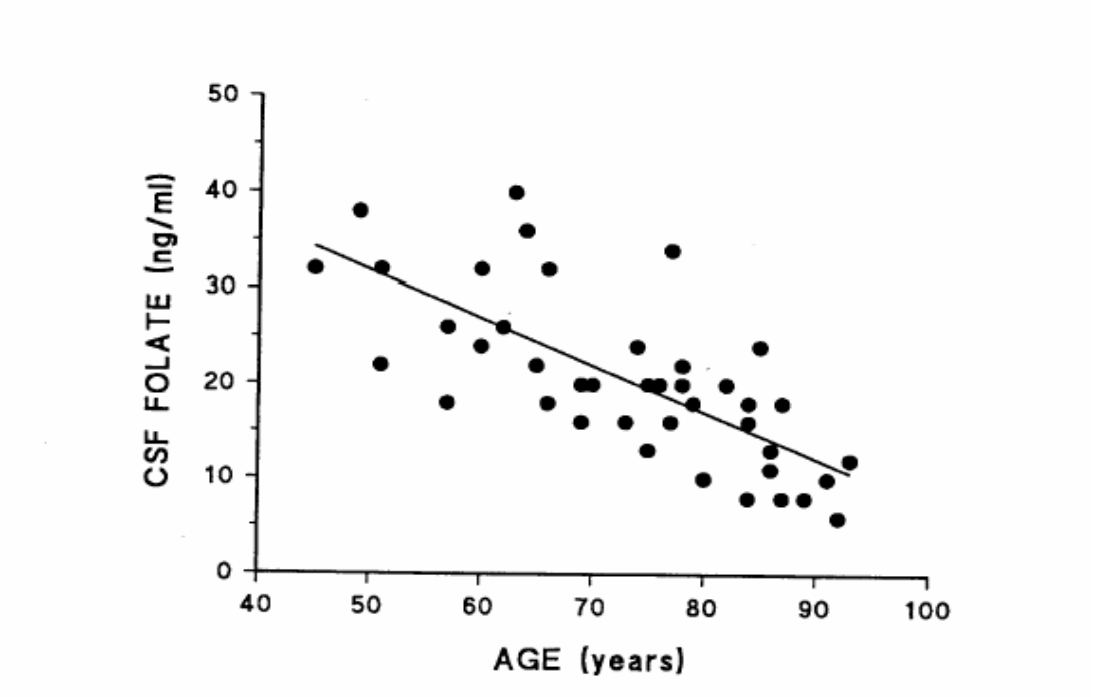


Figure 8. Correlation between age and CSF folate levels. Adapted from Bottiglieri et al. 2000.

affects the absorption of folate. The elderly population tends to have some health problems and as a result, is exposed to more medications that could possibly interact with folate metabolism/absorption/excretion. Thus, they are at a higher risk for folate deficiency than younger people. To summarize, elderly people are more at risk for folate deficiency due to increased risk for all three factors causing folate deficiency: disease, diet, and medication. The deficiency may be exacerbated in the presence of one of the many SNPs involved in folate metabolism.

Conditions of the Central Nervous System Associated with Folate Deficiency

There are many different pathological states of folate deficiency. Inherited and acquired defects in folate metabolism can lead to severe neurological impairment, whereas nutritional and drug induced folate deficiency tend to cause less severe complications. Nonetheless, there are a variety of diseases of the CNS associated with folate deficiency. These include but are not limited to: depression, mental disturbances, stroke, epilepsy, NTDs, multiple sclerosis, AD and age related cognitive decline (Czeizel and Dudas 1992; Hibbard et al. 1965; Kim et al. 2008; Morris et al. 2008; Bottiglieri 1996).

Cerebral Folate Deficiency

Cerebral folate deficiency (CFD) is defined as any neurological syndrome associated with decreased CSF 5-MTHF levels in the presence of normal folate outside the CNS. CFD results from either disturbance in folate transport across the BBB or from increased folate turnover within the CNS. Several folate transport abnormalities have been identified in humans. Irregularities in the reduced folate carrier 1 and the family of

folate receptor proteins have both been shown to be associated with CFD. However, some CFD patients have no abnormalities in genes encoding for folate receptors. This group of CFD patients was proven to have circulating antibodies that block the binding of folate to its anchored receptor, thus blocking folate transport into the CNS (Rothenberg 2004; Ramaekers and Blau 2004).

Depression and Psychiatric Disorders

One third of all psychiatric patients show signs of low or deficient folate status in plasma or RBC with incidences as high as 30% in patients with depression and schizophrenia (Carney 1967; Morris et al. 2003; Tiemeier et al. 2002; Young and Ghadirian 1989; Bottiglieri 1996). The correlation between low folate status and psychiatric disorders is highest among patients with depression (Carney et al. 1990). Additionally, the severity and duration of depression has been significantly correlated to serum folate levels (Leaitt and Joffe 1989). Furthermore, low folate status linked to depression is even more prevalent in the elderly population (Reynolds 2002; Bottiglieri et al. 1995). A study among 1,171 elderly individuals showed that those with low RBC folate levels were more likely to be depressed than those with satisfactory levels (Ebly et al. 1998). Even more convincing that folate levels play a role in psychiatric illness than the previously mentioned studies are studies that supplement folate to patients with psychiatric disorders. Various placebo-controlled double-blind studies have administered folic acid supplements to depressed patients alone or in combination with antidepressants and have reported substantial improvements in depressive symptoms, and social adjustment along with a reduction in Hamilton Rating Scale (Godfrey et al. 1990; Guaraldi et al. 1993; Passeri et al 1993; Coppen and Bailey 2000; Alpert et al. 2002;

Taylor et al. 2004; Coppen and Bolander-Gouaille 2005). The means by which folate influences the dopaminergic and serotonergic systems in depression are not conclusive, although several mechanisms have been proposed. One mechanism involves reduced monoamine neurotransmitter function as a result of an underactive folate dependent salvage pathway for the synthesis of BH_4.

Cognitive Impairment and Dementia

In 2003, Miller published mini-mental state exam (MMSE) scores on over 2,000 elderly people ranging from 60-80 years old. The study demonstrated a progressive age-related cognitive decline determined by MMSE scores, with the 80 and over group performing the worst (Miller et al. 2003). There is substantial epidemiological evidence linking low folate status to decline in cognitive function reported in the elderly (Kristensen et al. 1993; Ebly et al. 1998; Nilsson et al. 1999; Snowdon et al 2000; Bottiglieri et al. 2001; Selley et al. 2002; Ramos et al. 2005; Luchsinger et al. 2007; Metz et al. 1996; Troen and Rosenberg 2005). Low and deficient folate levels have been reproducibly and remarkably proven to be associated with diseases of the aging brain, including cognitive decline and dementia (Riggs et al. 1996; Budge et al. 2000; Bottiglieri et al. 2001; Kado et al 2005). Specifically, a long term study of over 300 Boston area men with no major health problems were administered cognitive tests upon entering the study and then again 3 years later; the study concluded that those who had sufficient folate levels improved on the cognitive test whereas those who were deficient performed significantly worse on the test the second time around (Tucker et al. 2005). Similarly, the NHANES study revealed participants in the 50^{th} percentile of plasma folate level had better memory test scores than those in lower percentiles (Morris et al. 2001).

A Swedish study that followed 370 non-demented geriatric subjects for 3 years reported low baseline levels of folate were correlated with risk of developing dementia (Wang et al. 2001). There are several mechanisms thought to be responsible for the prevalence of cognitive decline related to suboptimal folate levels: increased incorporation of dUTP into DNA; increased activation of NMDA receptors; increased oxidative stress; decreased methylation, or the potential ability of elevated Hcy to cause vascular disease that may result in brain ischemia (Selhub et al. 2000).

Other Neurological Disorders

Several studies indicate that folate deficiency may be associated with multiple sclerosis, restless leg syndrome (RLS), and autism. Reduced CSF folate levels have been reported in patients with multiple sclerosis (Nijst et al. 1990; Reynolds et al. 1992). Patients with RLS typically have normal RBC and serum folate levels, although a small cohort has been shown to have deficient CSF levels. Folic acid supplementation improved the symptoms associated with RLS in all patients, regardless of folate status prior to treatment (Patrick 2007). Many researchers are also currently working to determine whether there is a link between folate deficiency and autism due to the increase of autism cases since folic acid fortification (James et al. 2006). It is unclear if this correlation is the result of folic acid fortification, a coincidence, or an artifact of increased correct diagnosis. Interestingly, a SNP of DHFR has been established as a risk factor for autism (Adams et al. 2007). A systematic review of past literature concluded that further research is required before a definitive answer can be made about the role of folate and its metabolism in the etiology of autism (Main et al. 2010).

Aims of Dissertation

The aim of this dissertation is to characterize the effect of folate deficiency in the aging brain on metabolites of the methylation cycle and synthesis of neurotransmitters, using an aged C57BL/6J mouse model. Two methods of sacrificing mice were employed in this study: carbon dioxide (CO_2) asphyxiation and focused microwave radiation. This latter method of sacrificing mice inhibits post-mortem metabolism by inactivating enzymes within a few seconds. The microwave radiation technique was first developed to accurately quantitate brain concentrations of high energy phosphates such as adenosine triphosphate (ATP). It is also essential to determine metabolites such as: choline, acetylcholine and adenosine (Moroji et al. 1977; Schmidt et al. 1972; Stavinoha et al. 1973). The use of microwave radiation has never been used to study metabolites of the folate and methylation cycle. This novel approach will be fully investigated in this dissertation in a folate deficient mouse model.

The transgenic MTHFR (*tg*-MTHFR) mouse model will also be studied to determine if, in humans, MTHFR TT genotype exacerbates any of the metabolic and neurochemical changes caused by folate deficiency. In addition the effect of folate deficiency on behavior will also be investigated in both mouse models. The specific aims of this dissertation are as follows:

1) To characterize the effect of age on metabolites of the methylation cycle in C57BL/6J mice sacrificed by CO_2 asphyxiation.
2) To determine the effect of folate deficiency on metabolites of the methylation cycle in young and old C57BL/6J mice, and old *tg*-MTHFR mice using two different sacrifice techniques.

3) To determine the effect of folate deficiency on neurotransmitter metabolism in young C57BL/6J mice sacrificed by microwave radiation.
4) To determine if neurochemical changes related to folate deficiency in *tg*-MTHFR and C57BL/6J mice are associated with changes in behavior (sensorimotor and cognition).

The studies presented in this dissertation will help to further our understanding of the mechanisms involved in folate deficiency on CNS function in the aging brain, particularly as it relates to coordination and cognitive function.

CHAPTER TWO

Materials and Methods

Chemicals and Solvents

Chemicals

All chemicals were of high grade and were used in assays, either as reagents or as standards, Type 1 water was used throughout. All standards and stable isotopes used will be addressed within their prospective method section.

Solvents

Acetonitrile (ACN, Optima LC/MS), methanol (MeOH, Optima LC/MS), 75% perchloric acid solution (PCA), and 85% phosphoric acid solution were obtained from Fisher (Fair Lawn, NJ). Ethanol (EtOH) was purchased from Maiinckrodt Baker Inc. (Phillipsburg, NJ).

Animals and Experimental Designs

C57BL/6J Aged Folate Deficient Study Design

Young male C57BL/6J mice (3 months old) were obtained from Jackson Laboratories (Bar Harbor, ME). Aged male C57BL/6J mice (16 months old) were purchased from the National Institute of Aging (Bethesda, MD). Upon arrival each mouse was ear punched for identity purposes. The mice were housed together, never exceeding five per cage with a 12 hour light/dark cycle for at least 1 month prior to introduction to experimental diets. Mice were allowed access to water and food *ad*

libitum. Each cage was equipped with a tunnel and chew bone to provide an enriched environment. Five specific diet formulations were used in this study: normal chow diet (NCD); amino acid defined control diet without succinylsulfathiazole (SSA); amino acid defined control diet with SSA (CD); amino acid defined low folate diet (LF); or amino acid defined folate deficient diet (FD) (Table 2). All amino acid defined diets (AADD) were obtained from Harland Teklad (Madison, WI). Colored dyes were added to each amino acid defined diet during manufacturing for visual recognition. During the 3 months of treatment the mice were tested on several different behavioral tests, which will be discussed further in their forthcoming method sections.

Table 2. Nutritional composition of diets.

		Amino acid defined diets			
	NCD	CD (-SSA)	CD (+SSA)	LF (+SSA)	FD (+SSA)
Ingredient			g/kg		
Succinylsulfathiazole	0.0	0.0	10.0	10.0	10.0
Folic Acid	0.004	0.0067	0.0067	0.0002	0.0
Vitamin B12 (0.1% Mannitol)	0.00008	0.025	0.025	0.025	0.025
Pyridoxine HCL (82.3% B6)	0.018	0.0085	0.0085	0.0085	0.0085
Choline Bitartrate	1.2	2.0	2.0	2.0	2.0

After the completion of treatment, half the mice were sacrificed by CO_2 asphyxiation, and the other half by focused microwave radiation using the Gerling Applied Engineering system (Modesto, CA). This technique rapidly heats the brain and causes enzymatic inactivation, therefore preventing post-mortem changes in metabolites with high turnover rates. The mice are placed in a tight Plexiglas cylinder specifically designed for the instrument, that helps stabilize the mouse's head during radiation. The settings of the Gerling microwave were 4 KW for duration of 0.9 seconds. Blood was

drawn from asphyxiated mice by cardiac puncture using a tuberculin syringe containing ethylenediaminetetra acetic acid (EDTA), purchased from Sigma (St. Louis, MO) as an anticoagulant. Blood was immediately transferred to a 1.5 ml eppendorf tube and centrifuged for 20 minutes at 6000 rpm. Plasma was removed and stored at -80°C until analysis. Liver, kidney, and heart tissues were also collected from the asphyxiated mice. Peripheral tissues were removed and immediately frozen. A small portion of the peripheral tissues were rapidly deproteinized with ice-cold 0.4 M PCA (1:10 liver, and 1:5 kidney and heart) and homogenized. The tissue extracts were stored at -80°C until analysis. Additionally, six brain regions (striatum (STR), hippocampus (HIP), frontal cortex (FCX), cortex (CX), mid-brain (MB), and cerebellum (CB)) from both the left and right hemispheres (for twelve total) were rapidly dissected over ice from both asphyxiated and microwave radiated mice. Immediately, prior to HPLC analysis brain tissues from the left hemisphere were deproteinized either 1:10 (striatum, hippocampus, and frontal cortex) or 1:5 (cortex, cerebellum, and mid-brain) with ice-cold 0.1 M PCA containing 1 M Dithioerythritol (DTE) and 0.1 M Diethylenetriaminepentaacetic acid (DETAPAC) and centrifuged at 14000 rmp for 15 minutes. Right halves were stored for future additional analysis (Figure 9).

tg-MTHFR Aged Low Folate Study Design

Tg-MTHFR breeder mice were provided by Texas A&M Institute of Biosciences and Technology, thanks to Dr. Richard Finnell (University of Texas, Austin, TX). The MTHFR deficient mouse was produced by Dr. Rima Rozen (Montreal, Canada) through the targeted disruption of the MTHFR gene involving an insertion type of targeting vector, in which exon three of the mouse MTHFR gene was interrupted by the *neo* gene

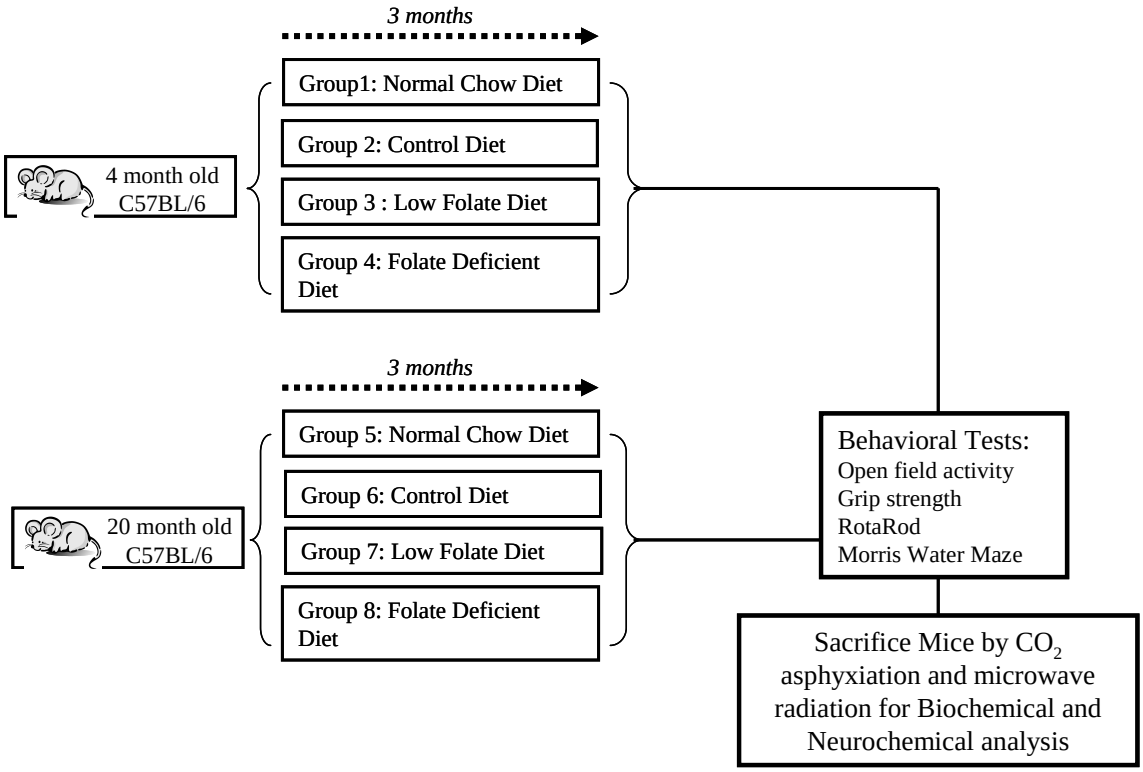


Figure 9. C57BL/6J aged folate deficient study design.

(Chen 2001). Homozygous mice have virtually no MTHFR activity while heterozygotes maintain 60-70% specific activity of the MTHFR gene compared to wild type (Dr. Erland Arning, Institute of Metabolic Disease, Dallas, TX, data not published). *Tg*-MTHFR mice were maintained in the same conditions as the C57BL/6J mice. Two of the specific diets were used in this experimentation: amino acid defined control diet and amino acid defined low folate diet (Table 2). At approximately 14.6 months of age treatment began and lasted for 6 months (Figure 10).

During treatment the mice were assessed on four different behavioral tests, which will be discussed in following sections. After experimentation all mice were sacrificed by CO_2 asphyxiation and plasma and tissues collected as previously mentioned. All

experiments involving mice within this dissertation were approved by the Institutional Animal Care and Use Committee at Baylor Research Institute (Dallas, TX).

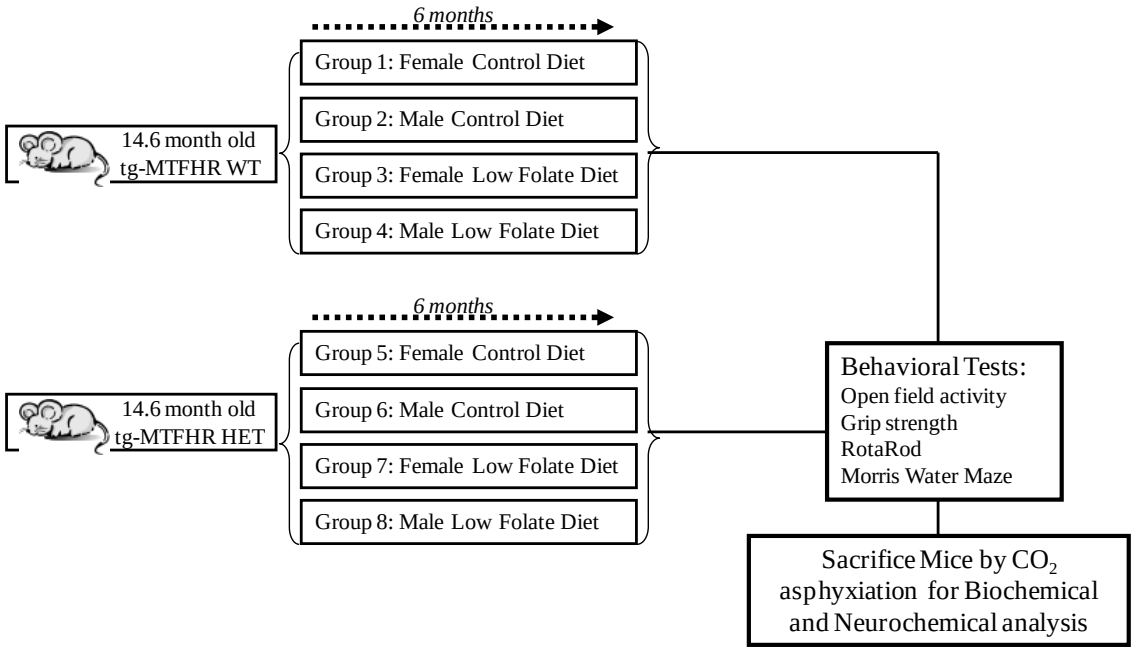


Figure 10. *tg*-MTHFR aged low folate study design.

Behavioral Methods

Grip Strength

The Chatillon grip strength meter was purchased from Columbus Instruments (Columbus, OH). It is used to assess neuromuscular function in small laboratory rodents by recording the peak amount of force the animal exerts in grasping a grip bar placed at their fore- or hindlimbs. Measurements are performed with precision force gauges Chatillon that are interfaced to the Grip Strength software (Nexygen DF Version V1.0), and the data (gf) collected and recorded on the computer. The grip strength meter consists of a base plate with a pedestal that supports the digital force gauge for a single

stand model. This arrangement allows for the meter to be positioned horizontally and raised in the air (Figure 11).

Figure 11. Grip strength meter apparatus.

Before neuromuscular function was evaluated, all mice were allowed to acclimate 1 hour in the testing environment prior to grip strength testing. For this dissertation the forelimb grip strength was measured using a single triangular wire grip. The animals were lowered by their tail to allow them to grasp the triangular grip and then were gently pulled backwards in the horizontal plane. The tension applied to the grip during the trial was recorded until the animal lost its grip. This was repeated five consecutive times with the maximum peak tension recorded for each trial, trials were averaged for analysis. Upon completion of the fifth trial the mouse was returned to its home cage.

Rota Rod

AccuScan Instruments Inc. (Columbus, OH) manufactures EzRod Accelerating rota rod chambers, which are used to test motor coordination and balance in mice. Each fully enclosed chamber is 13.97 cm wide, 43.18 cm deep, and 50.80 cm tall equipped with a 3 cm diameter 10.92 cm long rotating rod located 34.93 cm above the chamber floor (Figure 12). The rod has a ridged surface that assists the mouse's ability to grip the rotating rod, which can be accelerated up to 100 rpm. Fear of falling motivates the mice to stay on the rod during the duration of the test. The EzRod system allows trials to terminate based on one of two criterions: when the animal falls off the rod breaking the infrared beams at the base of the system, or when a no fall event has occurred (the animal completed the given task). During the experiment, EzRod 3.06 computer software is used to record data from six independent rota rod chambers.

Figure 12. Rota rod apparatus.

Mice were allowed to acclimate 1 hour in the testing environment prior to rota rod testing. Rota rod testing consists of two separate protocols: training session and testing session. The training session is to familiarize the mice with maintaining its position and

balance on the rotating rod. Mice were placed on the rotating rod at a speed of 4 rpm; the speed remained constant for 5 minutes followed by a linear deceleration to 0 rpm. If the mice fell during the training session, they were placed back on the rod until the 5 minutes had elapsed. Following the training session, mice were returned to their home cage to rest for 1 hour before beginning the testing session. The testing session consisted of three trials with 30 minute intervals between each trial. During a trial, mice were placed onto the rotating rod which linearly accelerated from 0 – 40 rpm over 5 minutes, followed by a 15 second linear deceleration to 0 rpm. A trial ended by either the mouse falling from the rod or remaining on the rod for the duration of the experiment. Mice were returned to their home cage between each trial. Both parts of this protocol were performed for three consecutive days and all data was collected into Microsoft Excel 2007. The two main parameters documented for this dissertation were latency to fall (seconds) and rpm at the time of fall. If a mouse completed the task the maximum rpm and time were recorded. All three trials of the testing session from each day were averaged for analysis, thus each test consisted of three data points.

TruScan®

TruScan® is a behavioral apparatus designed by Coulbourn Instruments' (Allentown, PA) to measure open field behavior in rodents; it is the most standardized general measure of motor function. The TruScan® system is a clear Plexiglas cage measuring 27.94 cm wide, 27.94 cm deep, and 38.10 cm tall. There are two adjustable 2.54 cm thick photo beam sensors E63-12 around the perimeter of the bottom of the cage (Figure 13). The system uses the two sensor rings to track the mouse in two dimensions. For the studies presented in this dissertation the lower floor plane (FP) ring was

positioned at the base of the cage and the upper vertical plane (VP) ring at 7.62 cm from the base of the cage. The precision is 32 x 32 twice the cage's beam resolution, with a beam spacing of 0.76 cm. The mice are tracked by these infrared beams arranged in a crisscross pattern. Unlike earlier instruments used to track open field behavior in laboratory animals, the TruScan system tracks the coordinates of the body center, thus, generating data with greater precision. The lower beam monitors movement in the XY plane (FP) by coordinate changes causing breaks in the grid pattern whereas the upper beam monitor breaks in the Z plane (VP). There are more than eighty parameters that can be measure by these two planes.

Figure 13. TruScan apparatus.

Six indistinguishable TruScan® apparatus were used during the course of experiments. In order to make the cage and room appear more uniform, the outside walls of the cage were covered with white copy paper, except for the area occupied by the

photo beams, thus not allowing the mice to see one another or outside stimuli. During the studies, five observations in the floor plane and one observation in the vertical place were recorded and converted into data for each run (Table 3).

Table 3. Open field behavior parameters assessed.

Plane	Parameter	Definition
Floor		
	FP Moves	Total movements in the FP. Each movement is a series of successive coordinate changes with no rest for at least 1 sample interval.
	Distance	The sum of all vectored coordinate changes in the FP.
	Jumps	The total number of time-contiguous 0-0 coordinate sets which do not exceed 2 seconds.
	Velocity	The mean speed of all the FP coordinate change defined movements.
	Stereotypic-Moves	The total number of coordinate changes less than plus or minus 0.999 beam spaces in each FP dimension and back to the orginal point that do not exceed 2 seconds apart. Three such movements must occur before considered a stereotypic action.
Vertical		
	VP Entry	Total number of times any part of the mouse penetrates the VP plane.

For experimentation the mice were placed in the TruScan® apparatus' at noon and their behavior was monitored continuously until 1:00 PM. Data was collected in 1 minute intervals resulting in sixty data points for each parameter. All data was totaled and averaged to obtain a single measurement for each parameter during the hour of testing.

Morris Water Maze

The Morris water maze (MWM) is the most frequently used paradigm to evaluate learning and memory in rats and mice. Typically, this is a spatial navigation task in which the subject is placed in a small pool of water facing the side of the pool to avoid bias and back-end first to avoid stress. The animal then swims to find a submerged

Plexiglas 10.5 cm diameter escape platform located in one of four quadrants of the pool using distinguished spatial clues around the examination room (Figure 14). Escape from the water and retrieval by the experimenter are the positive reinforcements. This test is based upon the principle that rodents are highly motivated to escape from the water in the fastest way possible; swimming directly from their start position to the platform in a straight path. During their search various parameters are recorded by a tracking camera mounted on the ceiling above the pool including: swim path, time spent in each quadrant, average swim speed, latency to reach platform and percent of time spent around the edge of the pool commonly referred to as thigmotaxis.

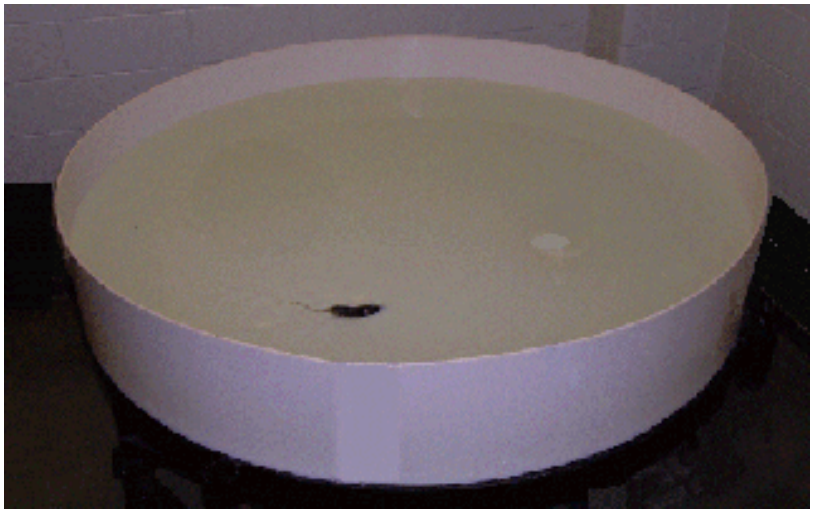


Figure 14. Morris water maze pool.

There are three different sessions to the protocol: a cued training session, a hidden session, and a probe session. Each of these sessions used the same varied start locations, but differing platform locations. During the cued training, the platform is raised 2 cm above the water level and a Styrofoam purple square on a 4 in wooden stick is used as a beacon for the mice, without spatial wall clues. This portion of testing is important to

ensure the mice are not visually impaired and have the will/ability to swim. The second session of testing requires the platform to be moved to a different location and submerged 2 cm below the surface of the water without a platform clue. Colorful geometrical patterns are hung on three walls around the experiment room to be used as spatial clues. The fourth wall or barrier was a hanging curtain matching the paint color of the other walls, which the experimenter was hidden behind during testing. This is when the learning and memory of the mice are evaluated. Lastly, a final probe session is given where the platform is removed, and the clues remain. A probe session assesses the ability of the subject to identify the spatial location that previously contained the hidden platform.

All water maze equipment was purchased from Coulbourn Instruments (Allentown PA). All experiments were carried out in a white 172.72 cm diameter pool that was 76.20 cm deep. The pool was filled to a depth of approximately 40 cm with 22-24°C tap water made opaque by the addition of nontoxic white Crayola paint. The platform was submerged in the southwest or northeast quadrant for cued trials and in the northwest or southeast quadrant for hidden trials and the probe trials, depending on the time of testing. During all sessions of the water maze, the start locations varied but were all equal distant from the platform and in the same order for each test subject. On each day of testing, mice were allowed to acclimate 1 hour in the testing environment prior to water maze testing. The first 4 days of testing consisted of cued training sessions. Throughout these 4 days, each subject was placed gently in the pool backend first facing the side of the wall at one of two start locations and allowed 90 seconds to locate the visual platform, while the examiner stood quietly behind a curtain out of view. When the

mouse reached his escape route, he remained there for 15 seconds before being removed to a holding cage lined with paper towels and positioned near a small heater. After the time elapsed if the mouse had not found the platform, he was guided there and remained for 15 seconds and then removed to the holding cage. This was repeated three times daily with a 2 minute interval between trials for four consecutive days. Day five of testing commenced the hidden trials. The hidden session also required three trials a day using the same procedure as cued trials except: testing lasted 6 days, the platform is moved and submerged 2 cm, and visual clues are hung on three walls within the room. On the eleventh day, one final trial was given, the probe trial. The platform was removed and the mice were only allowed 30 seconds to swim. All sessions were tracked using HVS tracking system (Hampton, FL) interfaced to ActiMetrics software version 2.6. During the probe session the position of the removed platform was still present in the software. This is important because the probe session measures the time spent in the target quadrant, defined as the quadrant that previously contained the hidden platform.

Molecular Techniques

tg- MTHFR Mice Genotyping

Every in-house *tg*-MTHFR mouse was tail snipped at 3 weeks of age for MTHFR genotype determination. DNA purification was performed using the DNeasy kit from Qiagen (Valencia, CA). The snip was approximately 0.4-0.6 cm long and placed in an autoclaved 1.5 ml microcentrifuge tube containing 180 µM buffer ATL (composed of 2.5-10% edetic acid and 2.5-10% sodium dodecyl sulphate) and 20 µM proteinase K. Sample was vortexed and incubated overnight at 56°C in a shaking water bath. The next

day, the sample was vortexed for 15 seconds, then 200 µl buffer AL and 200 µl ethanol were added and again immediately vortexed. The mixture was then transferred by pipette onto a DNeasy Mini spin column sitting inside a 2 ml collection tube and centrifuged at 8000 rpm for 1 minute. The filtrate was discarded and the DNeasy Mini spin column was placed in a new 2 ml collection tube. 500 µl of buffer AW1 was added and spun again using the same settings. This wash process was repeated again using buffer AW2 but at 14000 rpm for 3 minute in order to dry the DNeasy membrane. Finally, the column was placed in a new 1.5 ml sterile tube and 200 µl of buffer AE added, allowed to incubate at room temperature for 1 minute prior to centrifugation for 1 minute at 8000 rpm for DNA elution.

The region surrounding the mutation site was amplified with the primers 5'-GAAGCAGAGGGAAGGAGGCTTCAG-3' (endogenous MTHFR primer 1), 5'-AGCCTGAAGAACGAGATCAGCAGC-3' (MTHFR neo primer 2), and 5'-GACTAGCTGGCTATCCTCTCATCC-3' (common MTHFR primer 3), all of which were obtained from Sigma-Aldrich (St. Louis, MO). The endogenous and common MTHFR primers replicate the endogenous (i.e. wild type) region of the MTHFR gene, while the common and neo primers replicate the targeted disrupted (i.e. knock-out) region of the MTHFR gene. Polymerase chain reaction (PCR) amplification results in production of either an endogenous 145 bp fragment or a targeted 216 bp fragment.

Platinum blue PCR Supermix from Invitrogen (Carlsbad, CA) containing the taq polymerase, dNTPs, $MgCL_2$, and buffer was used for all PCR reactions. Each reaction mixture consisted of 45 µl Supermix, 1.5 µl each of the primers (200 nM), and 4 µl of DNA sample in a 0.2 ml tube. Samples were placed in the GeneAmp PCR system from

Applied Biosystems (Foster City, CA) and the program initiated. The PCR program consisted of thirty cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds followed by a final extension of 72°C for 10 minutes. In addition, a negative control (water blank) and positive control (heterozygote) were processed along with the unknowns. Following amplification, 10 µl of PCR product was loaded onto a 4-20% Novex gel from Invitrogen and ran at 150 mV for 35 minutes, immediately after genotype was determined.

mRNA Analysis

Total mRNA was isolated from liver tissues from young and old treatment mice using Trizol reagents following manufacture's instruction (Invitrogen). 2 µg of RNA was reverse-transcribed with reverse-transcriptase from Invitrogen. The relative mRNA levels for specific genes were then analyzed by quantitative PCR using SYBR Green on the ABI7300 system (Invitrogen). All mRNA analysis was performed courtesy of Dr. Jiandie Lin (University of Michigan).

Analytical Methods

5-Methyltetrahydrofolate

Plasma. Standard 5-MTHF was purchased from Schircks Laboratories (Switzerland) and $^{13}C_5$ 5-methyltetrahydrofolate ($^{13}C_5$ 5-MTHF) obtained from Merk Eprova. Dithiothreitol (DTT) and formic acid were purchased from (Fluka). HPLC system consisted of a complete Shimadzu Prominence binary HPLC system with a SCL-

10Avp controller. The mass spectrometer instrument used for analysis was an ABI 4000 QTRAP.

The method employed to quantitate 5-MTHF in plasma is stable-isotope dilution liquid chromatography-electrospray injection tandem mass spectrometry (LC-ESI-MS/MS). Stock solutions of 5-MTHF and the internal standard $^{13}C_5$ 5-MTHF were prepared in water with 1 mg/ml ascorbic acid at a concentration of 1 mmol/L and stored at -80ºC. A calibration curve was prepared by diluting stock solutions in Milli-Q water containing 1 mg/ml ascorbic acid 400, 200, 100, 50, and 25 nM for 5-MTHF. Sample preparation involved diluting 50 µl of standard, QC, or sample in 10 µl 62.5 mM DTT, 20 mg/ml ascorbic acid and 2.5 µM $^{13}C_5$5-MTHF. Sample was vortexed and incubated in the dark at room temperature for 10 minutes. Sample was deproteinized by the addition of 200 µl ACN containing 5 mg/ml ascorbic acid then vortexed and centrifuged at 14800 rpm for 10 minutes at 4ºC. Supernatant was removed and loaded in a 200 µl glass conical insert for LC-MS/MS analysis. The samples (20 µl) were injected on a Synergi Hydro 4µ 150x3mm maintained at 30°C (Phenomenex) and eluted in a gradient with buffer A (100% water with 0.1% formic acid) and buffer B (100% MeOH with 0.1% formic acid) with a flow rate of 0.5 ml/min. Gradient was initially set at 95%A:5%B from time 0 – 3.5 minutes and then 50%A:50%B from 2.6 – 6 minutes. After 6 minutes gradient returned to initial conditions and was allowed to re-equilibrate for 4 minutes, allowing for a total run time of 10 minutes. The flow from the column was delivered to the source from the period of 5 to 8 minutes; otherwise the flow was diverted to waste. The retention time 5-MTHF and $^{13}C_5$5-MTHF was 6 minutes.

The compounds were detected by multiple reaction monitoring (MRM) using positive ESI with a dwell time of 250 ms. The curtain gas was set at 25 L/min, and source gas 1 and 2 were set at 25 L/min. The heater was set to 300ºC with an ionspray voltage of 5500 V. Both compounds shared a lens potential was set to 10 V, declustering potential = 96, collision energy = 29, collision exit potential = 18, and the CAD gas (nitrogen) was set at 3.5 x 10e-5 Torr. The observed ions (m/z) values of the fragment ions were 5-MTHF (*m/z* 460→313) and $^{13}C_5$5-MTHF (*m/z* 465→313). All data were collected and processed using Analyst software version 1.4.2 (Applied Biosystems). Figure 15 shows representative HPLC chromatograms of a standard and plasma sample.

Total Homocysteine

Plasma. Determination of plasma tHcy was performed using reversed-phase HPLC with fluorescence detection (Shimadzu RF-10xL). Separation of Hcy and internal standard N-acetylcysteine (NAC) was achieved using a Phenomenex Synergi-Hydro Pro C18 150 x 3.0 mm 3µ column maintained at 40°C with an isocratic flow rate of 0.75 ml/min. The mobile phase consisted of 0.1 M sodium acetate, adjusted to a pH of 3.8 using concentrated acetic acid (approximately 40 ml). Detection of the compounds were performed with wavelengths set at excitation λ = 380 and emission = 516.

All standards, internal standards and chemical used within this assay were purchased from Sigma (St. Louis, MO). Standard and internal standard were made fresh daily. The final working standard and internal standard concentrations were 40 µM Hcy and 1.0 mM NAC. Stock solutions of 10% tri-*n*-butylphosphine (TNBP) in dimethylformamide (DMF) were also prepared daily under the hood by adding 450 µl

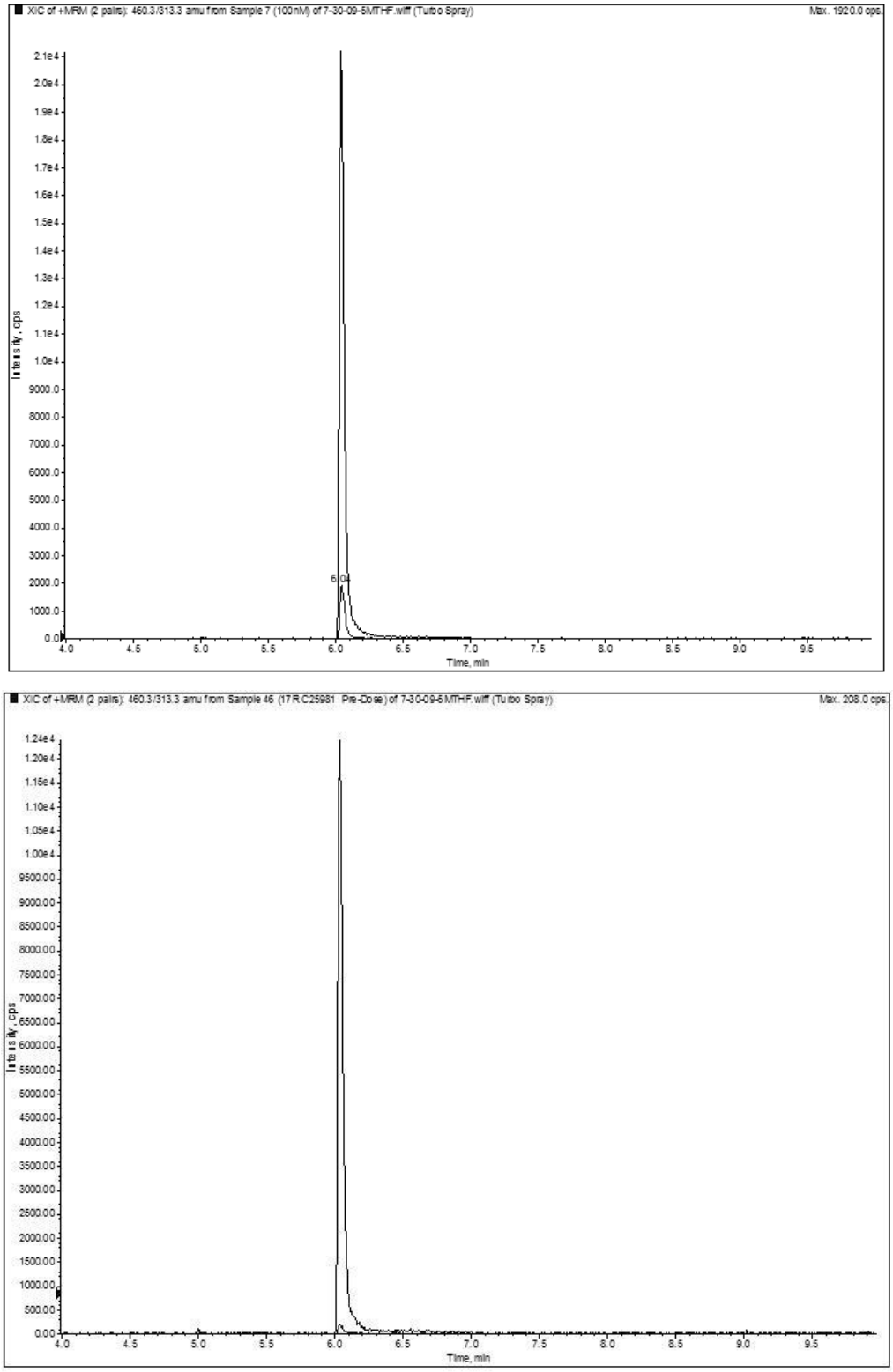


Figure 15. 5-MTHF LC/MS/MS chromatogram of standard and plasma sample. Top) 100 nM standard. Bottom) plasma sample from young low folate diet mouse.

DMF to 50 µl TNBP in a vial with a septum and vortexed. Stock solutions of 10% trichloric acid (TCA), 0.125 M borate buffer, 1.5 M sodium hydroxide (NaOH), and 13.8 mM 7-fluoro-2,1,3-benzoxaazole-4-sulfonamide (SBDF) are stable for a year, thus they were made when necessary.

First, 10 µl of plasma, standard, blank (H_2O), or QC was pipetted into an eppendorf tube and an equal amount of internal standard was added. The mixture was reduced by the addition of 1.6 µl TNBP and incubation for 30 minutes at 4°C. The reducing agent is necessary to cleave all disulfide bonds to liberate all Hcy; the free Hcy is what is measured. Next, 20 µl of 10% TCA was added, mixed, and spun in a microcetrifuge (14,000 rmp) for 5 minutes. Then 20 µl of clear supernatant was transferred to a new eppendorf tube. Borate buffer at a volume of 40 µl, and 4 µl of NaOH was added to the samples before they were derivatized by the addition of 4 µl SBDF and incubated at 60°C for 20 minutes. Following incubation 10 µl of sample was injected onto the HPLC system for analysis.

Monoamines and Their Metabolites

Brain tissue. Monoamines and their metabolites were analyzed using HPLC equipped with electrochemical detection. Dopamine, DOPAC, L-Dopa, 5-HIAA, HVA, 3-MT, and serotonin were eluted on a reverse-phase Phenomenex Gemini 5µ C18 250 x 3.0 mm column maintained at 35°C with a flow rate of 0.350 ml/min. The mobile phase was composed of 50 mM potassium dihydrogen phosphate, 1 mM octyl sodium sulfate (OSA), 54 µM EDTA, 14% methanol, and adjusted to pH = 2.65 with 85% phosphoric acid. Detection of the compounds was performed on an ESA Microdialysis cell 5014B

and ESA Guard cell 5020, with cell potentials set at E_1 = -10 mV, E_2 = +400 mV, and guard cell = 600 mV.

All standards and chemicals used in this assay were purchased from Sigma (St. Louis, MO) with the exception of OSA, which was acquired from Fluka (Seelze, Germany). Amine stock standards were prepared in 0.1 M HCL at a concentration of 1 mM and stored at -80°C. Calibration standards were diluted with ice-cold 0.1M PCA containing 1 M DTE, and 0.1 M DETAPAC to a final concentration of 1 μM each. 10 μl of sample or standard was injected straight onto the HPLC using a refrigerated autosampler.

Norepinephrine

Brain tissue. Norepinephrine was analyzed using HPLC equipped with electrochemical detection. NE was eluted on a reverse-phase Phenomenex Gemini C18 250 x 3.0 mm column with a particle size of 5 μm. The column was maintained at 35°C with a flow rate of 0.50 ml/min. The mobile phase was composed of 75 mM sodium dihydrogen phosphate, 1.5 mM sodium dodecyl sulfate, 25 μM EDTA, 5% MeOH, 20% ACN and adjusted to pH = 5.6 with 85% phosphoric acid. Detection of the compounds were performed on an ESA cell 5014B with cell potentials set at E_1 = +50 mV and E_2 = +250 mV.

Stock standard of NE was prepared in 0.1 M HCL at a concentration of 1 mM and stored at -80°C. Calibration standard was prepared by dilution of stock standard with a mixture of 0.1M PCA containing, 1 M DTE, and 0.1 M DETAPAC to a final

concentration of 10 nM. 10 µl of tissue PCA extracts and standards were injected straight onto the HPLC system.

Tetrahydrobiopterin

Brain tissue. Reversed phase chromatography using a Phenomenex Synergi Hydro C18 250 x 3 mm, 4 µm column was used for the analysis of BH_4. The mobile phase was composed of 0.5 M sodium acetate, 5 mM citric acid, 54 µM EDTA, and 0.65 mM DTE. Detection of BH_4 was performed on a Coulochem II with an ESA 5011A cell at cell potentials of, E_1 = -100 mV and E_2 = -400 mV.

Standard and all chemicals were purchased from Sigma (St. Louis, MO) BH_4 was stored as a stock solution at a concentration of 250 µM. On the day of analysis it was diluted to a final concentration of 125 nM using 1 M DTE, and 0.1 M DETAPAC, made fresh daily in 0.1 M PCA. 10 µl of standard and sample were injected straight onto the HPLC system at time of analysis.

Methylation Cycle Metabolites, Amino Acids, GABA, and Ach

Brain and peripheral tissue. Standards of SAM, SAH, acetylcholine, ADMA, betaine, choline, γ aminobutyric acid (GABA), glutamate, glutamine (GLN), methionine, SDMA, tryptophan, tyrosine, and adenosine were purchased from Sigma (St. Louis, MO, USA). Stable isotopes adenosine (ribose-5,5-d_2), asymmetric dimethylargininge-d7 (ADMA-d_7), glutamic acid (HOO*C(*CH_2)$_2$*CH(*NH_2)*COOH), glutamine (2,3,3,4,4-d_5), acetylcholine (1,1,2,2-d_4 Chloride), betaine (N-methyl-d_3-Cl), choline (1,1,2,2-d_4 Chloride), gama aminobutyric acid (3,3-d_2), methionine (S-methyl-d_3), tryptophan

(2,3,3-d_3), SAM (S-methyl-d_3), cystathionine (3,3,4,4-d_4) and tyrosine (4-hydroxyphenyl-d_4-alanine-2,3,3-d_3) were purchased from Cambridge Isotope Laboratories (Andore, MA) or CDN Isotopes Inc. (Quebec, Canada). Heptafluorobutyric acid and formic acid were purchased from (Fluka). Ammonium acetate was purchased from Sigma (St. Louis, MO) and Optima LC/MS MeOH from Fisher Chemicals. HPLC system consisted of a complete Shimadzu Prominence HPLC system with a SCL-10Avp controller. The instrument used for analysis was an ABI 4000 QTRAP LC/MS/MS system.

Three groups of stock standards were prepared in 0.1 M HCL at a concentration of 1 mM and fourth group was prepared in 0.1 M HCL at a concentration of 4 mM. All stable isotope internal standards were prepared in 0.1 M HCL at a concentration of 1mM and stored at -80ºC. All four calibration standard groups were stored at -80°C until needed. From these stock solutions a five level standard curve was prepared by diluting stock solutions in Type 1 water at concentration shown in table 4. Samples were prepared by the addition of 30 µL mobile phase A containing 20 µmol/L of each stable isotope internal standard to 10 µL of blank, standard, QC or tissue PCA extract. The sample mixture was vortexed and spun in a microcetrifuge (14,800 rmp at 4°C) for 5 minutes.

Table 4. Tissue standard curve concentrations.

		Curve Level (µm)				
Group	Compounds	1	2	3	4	5
1	ADE, ADMA, SDMA, SAH	0.063	0.125	0.3	0.5	1
2	CYSTA, MET, SAM, TRP	0.625	1.25	2.5	5	10
3	Ach, BET, CHO, TYR,	6.25	12.5	25	50	100
4	GABA, GLN, GLU,	62.5	125	250	500	1000

An injection volume of 5 µl was injected onto the LC-MS system by a Shimadzu Prominence LC System interfaced with a 4000 QTRAP® LC-MS/MS (Applied Biosystems). Solvents for HPLC were: A, 4 mM ammonium acetate, 0.1% formic acid, 0.1% heptafluorobutyric acid (pH=2.5); B 100% MeOH and 0.1% formic acid. Prior to MRM analysis all analytes were separated on a 250 x 2.0mm EZ-faast analytical column (Phenomenex) maintained at 33°C at a flow of 250 µL/min with a binary gradient with a total run time of 12 minutes. The initial gradient condition was 75% A: 25% B and was ramped in a linear fashion to 100% B in 6 minutes and held constant for 1 minute. At 7.1 minutes the mobile phase was reset to initial conditions for 7 minutes. The flow from the column was delivered to the ESI source from the period of 3 to 8 minutes; otherwise the flow was diverted to waste.

The compounds were detected by MRM using positive ESI with a dwell time of 30 ms. The curtain gas was set at 15 L/min, and source gas 1 and 2 were set at 60 L/min. The heater was set to 700ºC with an ionspray voltage of 5000 V and CAD gas (nitrogen) was set at 3.5 x 10e-5 Torr. Analyte specific MRM transitions monitored, declustering potentials (DP), entrance potential (EP), collision energy (CE), and collision exit potential (CXP) for all compounds are shown in table 5. All data was collected using Analyst software version 1.4.2 (Applied Biosystems). Validation of the in-house method is supported by tables 6 and 7. Correlation significance is $p < 0.0001$ for all compounds and the average recovery was 90%. Both intra- and inter-assays were generally had a CV of less than 10%. Figures 16 and 17 shows representative HPLC chromatograms of brain tissue sample and liver tissue sample.

Table 5. Analyte specific MRM transitions and detection parameters.

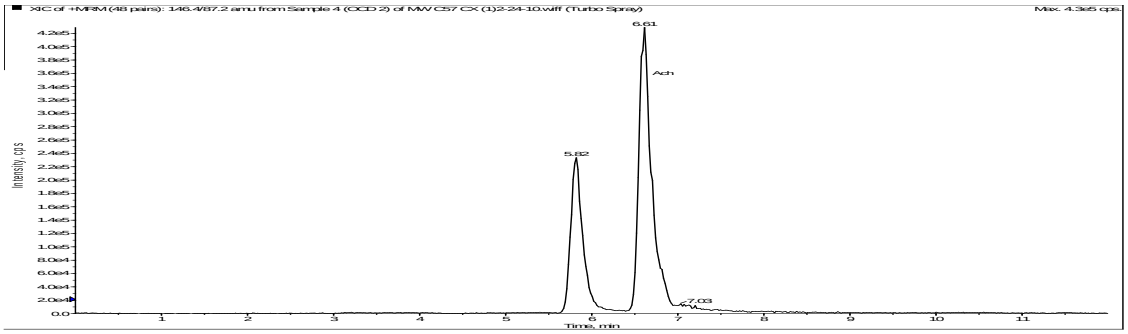
Compound	Abbreviation	Q1 MASS	Q3 MASS	DP	EP	CE	CXP
Acetylcholine	Ach	146.4	87.2	50	10	20	6
Acetylcholine-d4	Ach-D4	150.4	91.2	50	10	20	6
Adenosine	ADE	268.2	136	71	10	25	10
Adenosine-d2	ADE-D2	270.2	136	70	10	25	10
Asymmetric dimethylarginine	ADMA	203.7	46.2	66	10	39	6
Asymmetric dimethylarginine-d7	ADMA-D7	210.1	46.2	66	10	39	6
Betaine	BET	118.1	59.2	77	10	27	2
Betaine-d3	BET-D3	121.1	61.1	60	10	40	10
Choline	CHO	104.2	45.2	75	10	36	2
Choline-d4	CHO-D4	108.1	49.2	65	10	35	2
Cystathionine	CYSTA	223	134	46	10	21	8
Cystathionine-d4	CYSTA-D4	227.3	138.2	60	10	21	10
Gama-aminobutyric acid	GABA	104.1	87.1	36	10	15	6
Gama-aminobutyric acid-d2	GABA-D2	106.1	89.1	40	10	15	6
Glutamate	GLU	148	130	31	10	15	8
Glutamic Acid-c5n	GLU-C5N	154.4	89.2	45	10	25	2
Glutamine	GLN	147	130	36	10	15	10
Glutamine-d5	GLN-D5	152.2	81.4	31	10	22	6
Methionine	MET	150	104	41	10	15	6
Methionine-d3	MET-D3	153.3	107.1	30	10	15	8
S-adenosylhomocysteine	SAH	385	136.18	26	8	27	18
S-adenosylmethionine	SAM	399	250	31	6	23	16
S-adenosylmethionine-d3	SAM-D3	402	250	31	6	23	16
Symmetric dimethylarginine	SDMA	203.1	172.2	56	10	19	12
Tryptophan	TRP	205	188	50	10	17	10
Tryptophan-d3	TRP-D3	208.5	191.2	55	10	15	10
Tyrosine	TYR	182.1	136.2	60	10	19	10
Tyrsosine-d7	TYR-D7	189.3	143.1	55	10	20	10

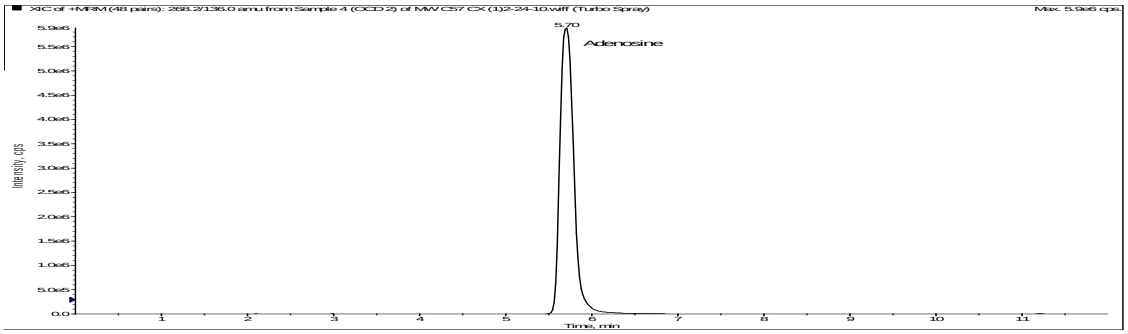
Table 6. Validation LC/MS/MS method: Correlation significance and percent recovery.

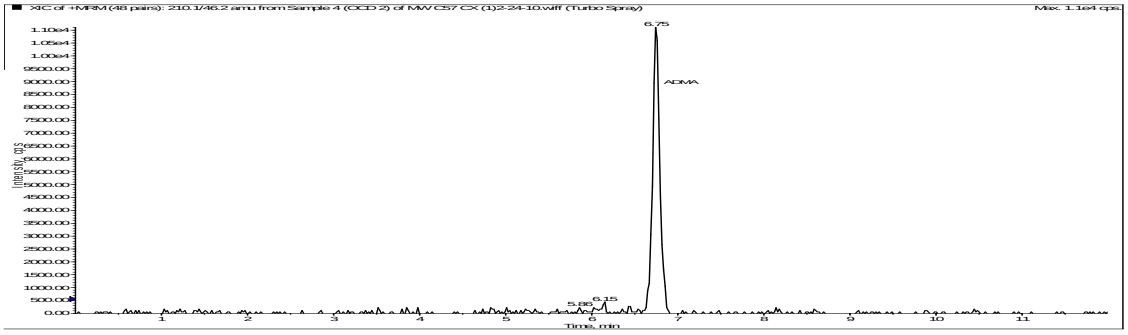
Compound	# of points	Lower Limit	Pearson R	Correlation Sig.	% Recovery
Ach	5	<5 µM	0.9861	p < 0.0001	99.21
ADE	5	<10 nM	1.0000	p < 0.0001	not avaliable
ADMA	5	<10 nM	0.9976	p < 0.0001	105.7
BET	5	<5 µM	0.9996	p < 0.0001	105.5
CHO	5	<5 µM	0.9999	p < 0.0001	88.9
CYSTA	5	<10nM	1.0000	p < 0.0001	94
GABA	5	<50 µM	0.9948	p < 0.0001	105.1
GLU	5	<50 µM	0.9961	p < 0.0001	109.1
GLN	5	<50 µM	0.9992	p < 0.0001	99.2
MET	5	<10 nM	0.9978	p < 0.0001	102
SAH	5	<10nM	0.9981	p < 0.0001	83.2
SAM	5	<5nM	0.9997	p < 0.0001	91.2
SDMA	5	<10nM	0.9998	p < 0.0001	105.4
TRP	5	<10 nM	0.9990	p < 0.0001	96.6
TYR	5	<5 µM	0.9999	p < 0.0001	60.3

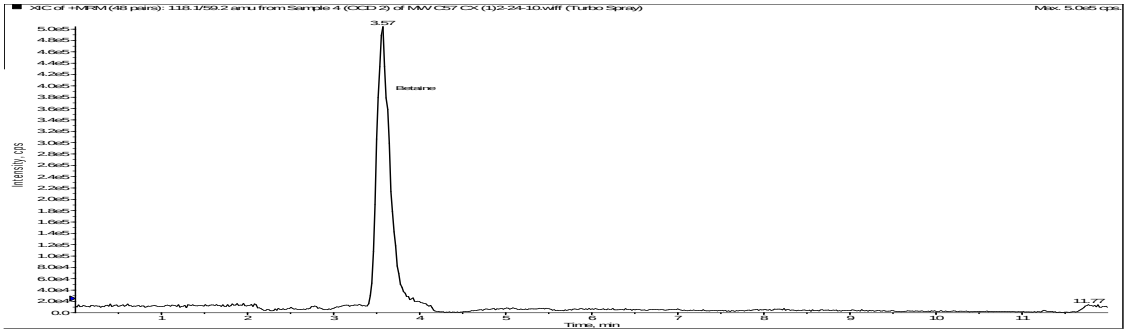
Table 7. Validation LC/MS/MS method: Quality control.

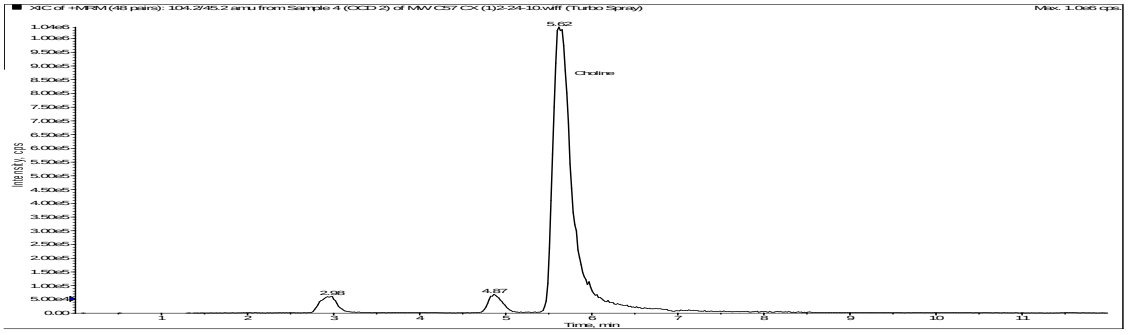
		Intra-Assay (CV%)	Inter-Assay (CV%)		
Compound	# of points	QC 1	QC 1	QC 2	QC 3
Ach	30	not avaliable	9.02	7.9	8.8
ADE	30	not avaliable	5.6	6.4	6.4
ADMA	30	15.6	10.9	10.0	8.8
BET	30	7.6	15.3	11.0	11.4
CHO	30	10.5	14.5	9.2	8.3
CYSTA	30	3.7	10.5	9.4	8.2
GABA	30	11.7	11.5	10.4	8.9
GLU	30	4.3	6.4	6.5	6.9
GLN	30	2.5	8	10.7	9.8
MET	30	10.1	9.8	8.7	9.2
SAH	30	13.2	9.7	9.7	9.9
SAM	30	4.9	9.8	8.4	7.9
SDMA	30	5.4	7.3	7.1	6.6
TRP	30	8.5	12	10.8	10.2
TYR	30	11.1	10.4	7.4	6.6

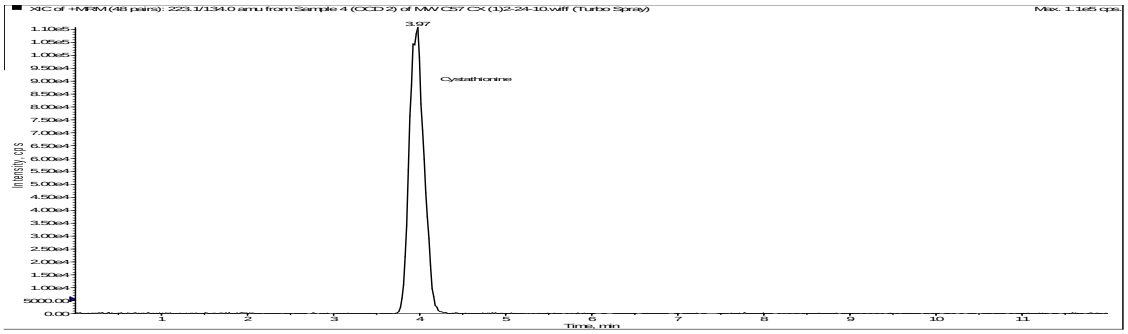

XIC of +MRM (48 pairs): 146.4/87.2 amu from Sample 4 (OCD 2) of MW CS7 CX (1)2-24-10.wiff (Turbo Spray)
Max. 4.3e5 cps.
Intensity, cps
Time, min
6.61
Ach
5.82
7.03

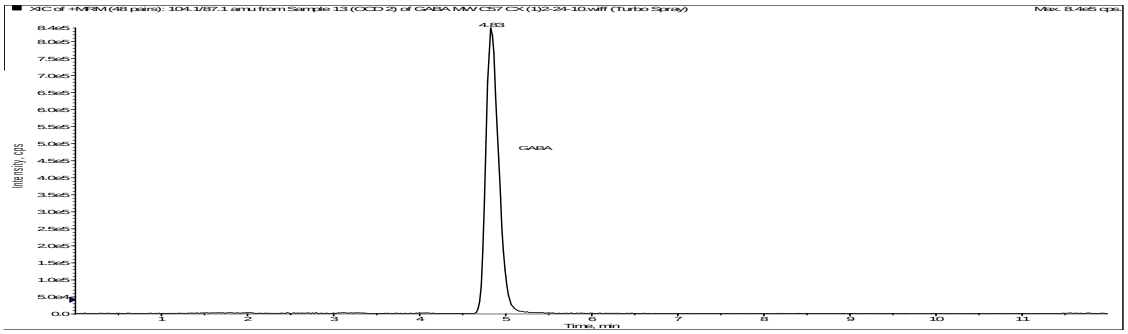

XIC of +MRM (48 pairs): 268.2/136.0 amu from Sample 4 (OCD 2) of MW CS7 CX (1)2-24-10.wiff (Turbo Spray)
Max. 5.9e6 cps.
Intensity, cps
Time, min
5.70
Adenosine

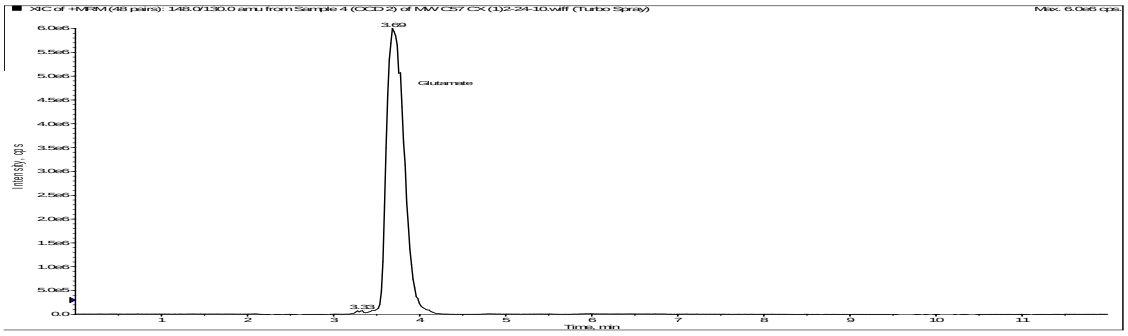

XIC of +MRM (48 pairs): 210.1/46.2 amu from Sample 4 (OCD 2) of MW CS7 CX (1)2-24-10.wiff (Turbo Spray)
Max. 1.1e4 cps.
Intensity, cps
Time, min
6.75
ADMA
6.15
5.85

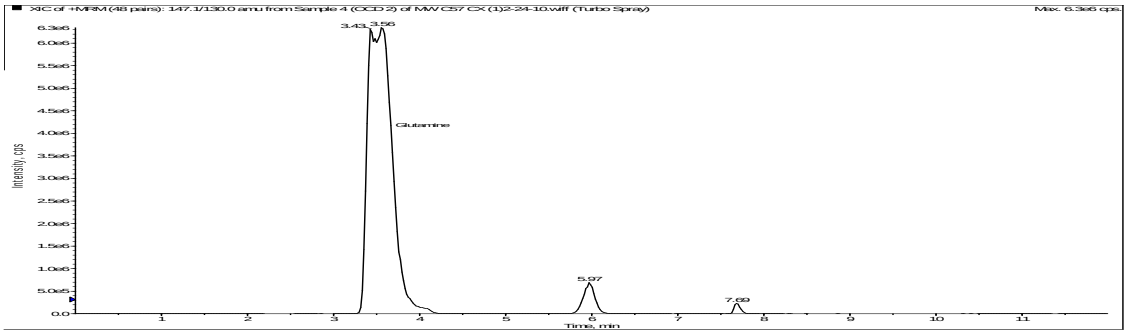

XIC of +MRM (48 pairs): 118.1/59.2 amu from Sample 4 (OCD 2) of MW CS7 CX (1)2-24-10.wiff (Turbo Spray)
Max. 5.0e5 cps.
Intensity, cps
Time, min
3.57
Betaine
11.77

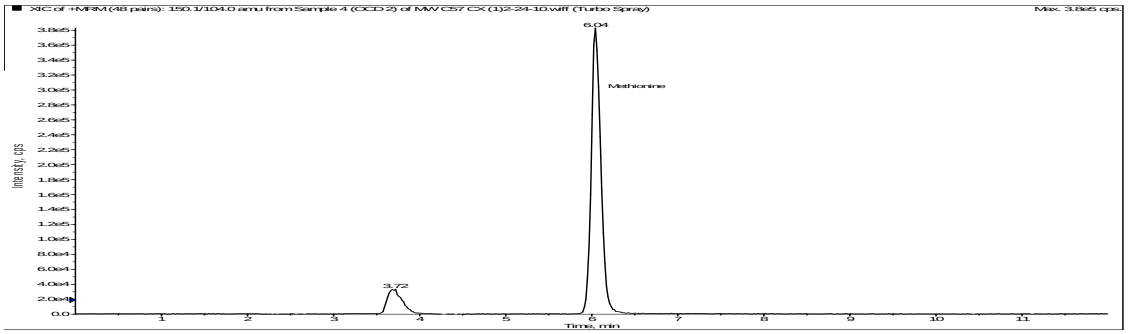

XIC of +MRM (48 pairs): 104.2/45.2 amu from Sample 4 (OCD 2) of MW CS7 CX (1)2-24-10.wiff (Turbo Spray)
Max. 1.0e6 cps.
Intensity, cps
Time, min
5.62
Choline
2.98
4.87


XIC of +MRM (48 pairs): 223.1/134.0 amu from Sample 4 (QCD 2) of MW C57 CX (1)2-24-10.wiff (Turbo Spray)
Max. 1.1e5 cps.
3.97
Cystathionine
Intensity, cps
Time, min


XIC of +MRM (48 pairs): 104.1/87.1 amu from Sample 13 (QCD 2) of GABA MW C57 CX (1)2-24-10.wiff (Turbo Spray)
Max. 8.4e5 cps.
4.83
GABA
Intensity, cps
Time, min


XIC of +MRM (48 pairs): 148.0/130.0 amu from Sample 4 (QCD 2) of MW C57 CX (1)2-24-10.wiff (Turbo Spray)
Max. 6.0e6 cps.
3.69
Glutamate
3.33
Intensity, cps
Time, min


XIC of +MRM (48 pairs): 147.1/130.0 amu from Sample 4 (QCD 2) of MW C57 CX (1)2-24-10.wiff (Turbo Spray)
Max. 6.3e6 cps.
3.43
3.56
Glutamine
5.97
7.69
Intensity, cps
Time, min


XIC of +MRM (48 pairs): 150.1/104.0 amu from Sample 4 (QCD 2) of MW C57 CX (1)2-24-10.wiff (Turbo Spray)
Max. 3.8e5 cps.
6.04
Methionine
3.72
Intensity, cps
Time, min

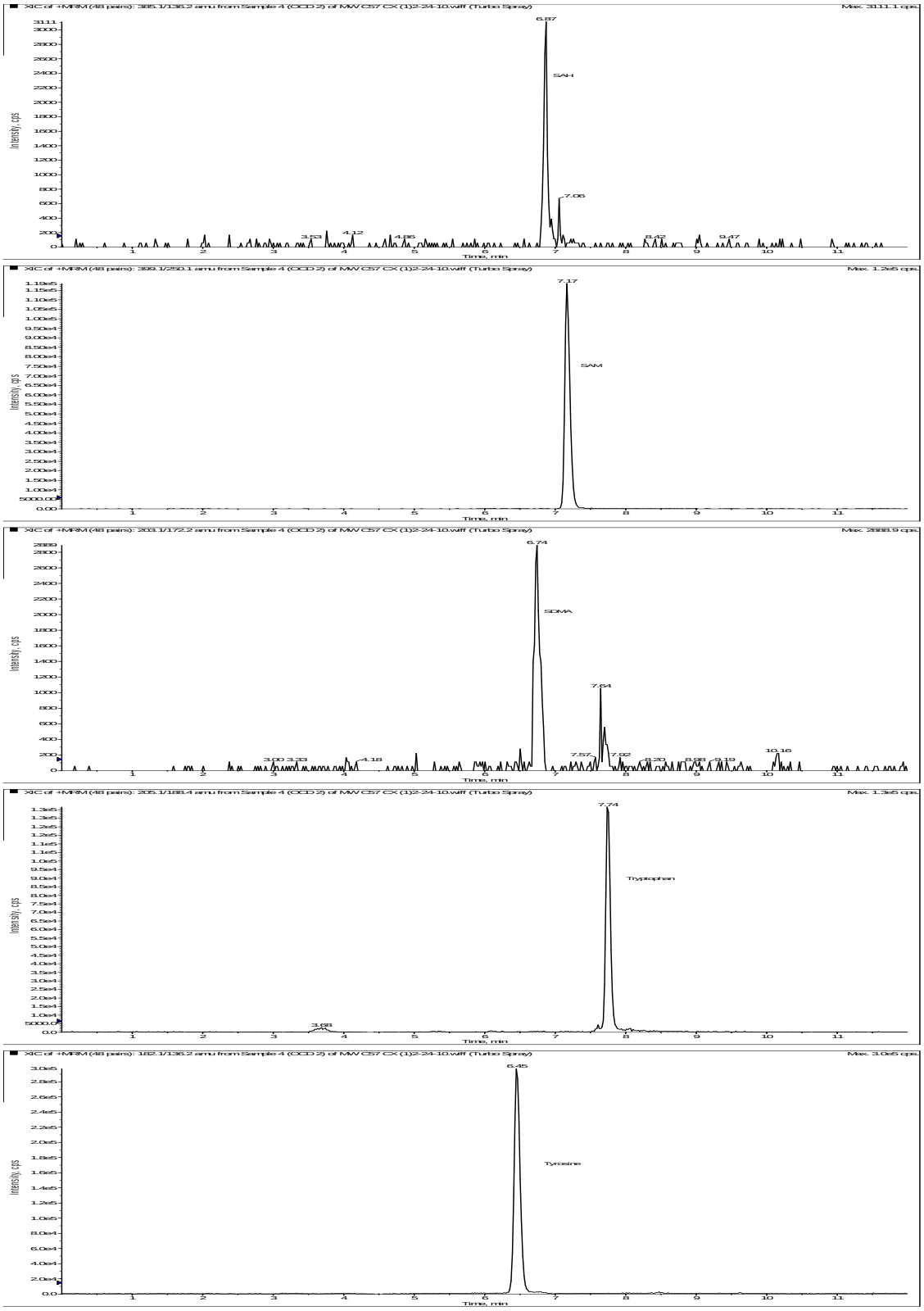
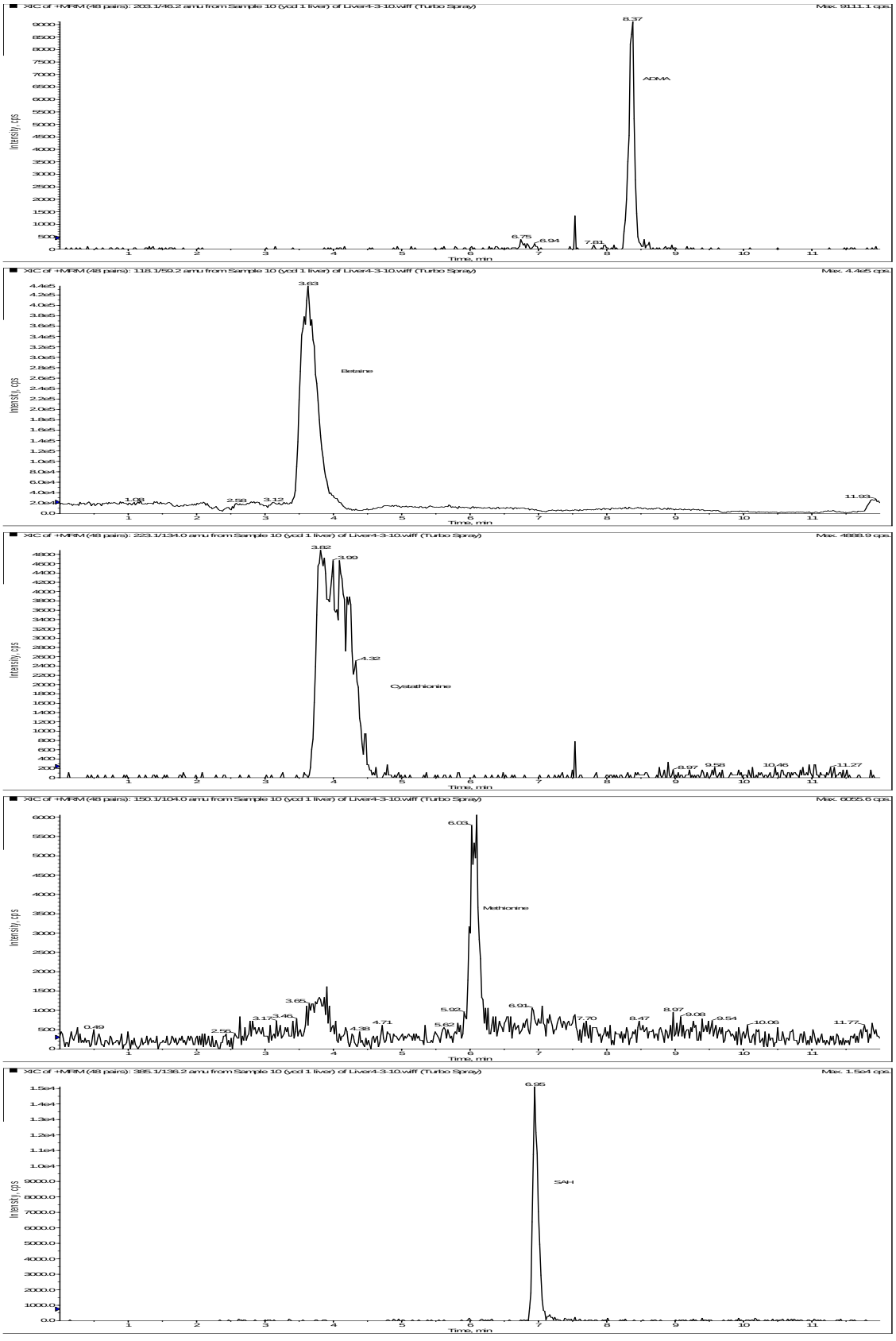


Figure 16. Methylation metabolites LC/MS/MS chromatogram of cortex from old control diet mouse.


XIC of +MRM (48 pairs): 203.1/46.2 amu from Sample 10 (ycd 1 liver) of Liver4-3-10.wiff (Turbo Spray)
Max. 9111.1 cps.
8.37
ADMA
6.75
6.94
7.81
Intensity, cps
Time, min
XIC of +MRM (48 pairs): 118.1/59.2 amu from Sample 10 (ycd 1 liver) of Liver4-3-10.wiff (Turbo Spray)
Max. 4.4e5 cps.
3.63
Betaine
1.68
2.59
3.12
11.93
Intensity, cps
Time, min
XIC of +MRM (48 pairs): 223.1/134.0 amu from Sample 10 (ycd 1 liver) of Liver4-3-10.wiff (Turbo Spray)
Max. 4828.9 cps.
3.82
3.99
4.32
Cystathionine
8.97
9.88
10.46
11.27
Intensity, cps
Time, min
XIC of +MRM (48 pairs): 150.1/104.0 amu from Sample 10 (ycd 1 liver) of Liver4-3-10.wiff (Turbo Spray)
Max. 6055.6 cps.
6.03
Methionine
0.49
2.59
3.17
3.46
3.65
4.38
4.71
5.62
5.92
6.91
7.70
8.47
8.97
9.08
9.54
10.08
11.77
Intensity, cps
Time, min
XIC of +MRM (48 pairs): 385.1/136.2 amu from Sample 10 (ycd 1 liver) of Liver4-3-10.wiff (Turbo Spray)
Max. 1.5e4 cps.
6.95
SAH
Intensity, cps
Time, min

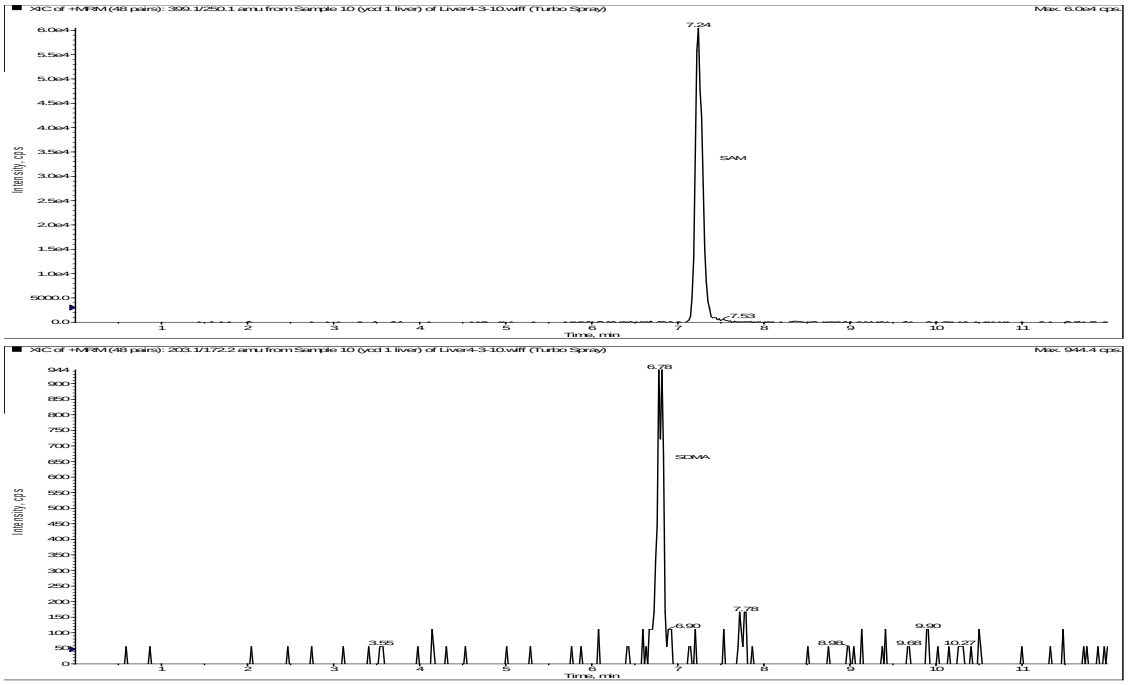


Figure 17. Methylation metabolites LC/MS/MS chromatogram of liver from old control diet mouse.

Plasma. LC/MS/MS conditions and settings used in analysis of methylation metabolites in plasma are identical to those described above. Plasma analysis included the following compounds: SAM, SAH, ADMA, SDMA, methionine, cystathionine, betaine, and choline. Samples were prepared by the addition of 30 µL mobile phase A containing 20 µM each stable isotope (same as tissue analysis) to 10 µL of standard or plasma to a Microcon YM-10, 10 kDa NMWL microcentrifugal filter unit and centrifuged for 15 minutes at 14800 x g at 4ºC. The plasma standard curve is presented in table 8. Sample filtrate was removed and transferred to autosampler vial for analysis. 5 µl was injected into the LC-MS system, a Shimadzu Prominence LC System interfaced with a 4000 QTRAP® LC-MS/MS (Applied Biosystems).

Table 8. Plasma standard curve concentrations.

		Curve Level				
Compounds	Units	1	2	3	4	5
SAM, SAH, CYSTA	nM	25	50	100	200	400
ADMA, SDMA	nM	250	500	1000	2000	4000
MET, Choline, Betaine	µM	2.5	5	10	20	40

Data and Statistics

Data was collected, managed, and stored in Microsoft Excel. Biochemical data was presented as mean ± standard deviation unless otherwise stated. Student's t-test was used for all biochemical analysis only consisting of two groups. Statistical analysis of biochemical data from three or more groups was performed used one way analysis of variance (ANOVA) with a post Dunnett's t-test. All behavioral data is represented as mean ± standard error of the mean unless otherwise indicated. The Student's t test and a one way ANOVA were used to determine statistical significance for all open field behavior data. Repeated AR-1 covariance matrices were performed on all rota rod and MWM data by Dr. Filardo Giovanni, director of epidemiology at Baylor and assistant professor of statistics at Southern Methodist University. These analyses took into account: weight, age, and swim speed, when necessary. Graph Pad Prism version 4.0 (Graph Pad Software Inc. San Diego, CA) was used for all data and graphs.

CHAPTER THREE

Evaluation of Age and Control Diets on Methylation Metabolites in Young and Old C57BL/6J Mice Sacrificed by CO_2 Asphyxiation

Introduction

Many age-related diseases in particular vascular disease, depression and dementia are associated with disturbances in folate metabolism and methylation (Bottiglieri et al. 1990; Morrison et al. 1996; Nile et al. 2008; Socha et al. 2009; Vanyushin et al. 1973; Wilson et al. 1987; Richardson 2003). SAM, a metabolite of the amino acid methionine is the major methyl donor in all methyltransferase reactions and produces SAH as the byproduct. The ratio of SAM/SAH has been proposed to be an indicator of methylation status, with a low ratio indicating a state of hypomethylation (Chiang et al. 1996). The reaction that links folate metabolism with methylation is catalyzed by MTR. MTR is regenerated via methionine synthetase reductase (MTRR). In this process a methyl group is transferred from 5-MTHF to Hcy to produce methionine. However, betaine can replace 5-MTHF and act as an alternate methyl donor for the conversion of Hcy to methionine. This salvage pathway is catalyzed by the enzyme BHMT. Activity of this enzyme is primarily located in liver and kidney, and is completely absent in brain tissues (McKeever et al. 1991; Sunden et al. 1997). Since the brain lacks the BHMT salvage pathway, it may be more susceptible to hypomethylation when folate levels are compromised. It has been suggested that methionine produced in peripheral tissues via the BHMT pathway can be utilized by CNS tissue through increased uptake from the circulation (McKeever et al. 1991). It is important to note that the only source of betaine

is from oxidation of choline, that is derived either from the diet or phosphatidylcholine and the choline (CDP) pathway (Crews 1982).

Many epidemiological studies have shown that there is an inverse relationship between the concentration of 5-MTHF and tHcy in plasma (Clarke et al. 1998; Miller 1999; Snowdon et al. 2000; Selhub et al. 1993; Boushey et al. 1995; Folsom et al. 1998; Giles et al. 1998; Bates et al. 1999; Jacques et al. 2001). Clinical studies have also shown that tHcy levels increase and 5-MTHF levels decrease with age; and this may be due to the fact that 60% of people over 51 years old have inadequate folate intake from their diet (Foote et al. 2000; Pennypacker et al. 1992; Bottiglieri et al. 2000; Serot et al. 2005; Rosenberg 2001). Thus, the primary focus of this chapter is to evaluate the effect of age on methylation metabolites in peripheral and CNS tissues in mice fed a normal chow diet and mice fed an amino acid defined control diet. A comprehensive analysis of methylation cycle metabolites which included methionine, SAM, SAH, choline and betaine was performed. There is evidence that the methylation cycle is down regulated and the transsulfuration pathway up regulated in aged animals thereby, the metabolite cystathionine of the transsulfuration pathway is also of particular interest (Finkelstein et al. 1975; Mays et al. 1973). In addition, levels of ADMA and SDMA were also investigated in various peripheral tissue and regional areas of brain tissue.

Folate deficiency is induced commonly in rodents by rearing on an amino acid defined diet void of folate. Age, strain, length on the diet, other nutritional components removed from the diet, and antibacterials, such as SSA are all variables that can determine the extent of folate deficiency in mice. SSA acts as a competitive inhibitor of dihydropteroate synthetase (DHPS), an enzyme involved in bacterial folate synthesis.

When added to the diet, SSA prevents the growth of folate absorbing cecal bacteria in the gut thus preventing folate absorption from this source. Therefore, secondary focuses of this chapter are to evaluate the effect of length of treatment, addition of SSA, and control diets on methylation metabolites in peripheral and CNS tissues in young and old mice.

Results

Impact of Age and Type of Control Diet on Weight

Mice reared on an amino acid defined control diet did not have any growth abnormalities during the 3 month treatment period compared to mice fed a normal chow diet (Figure 18). The increased weight associated with age was not significantly different between the normal chow and amino acid defined control diets (Figure 18). Overall, all groups did not gain or lose weight during treatment.

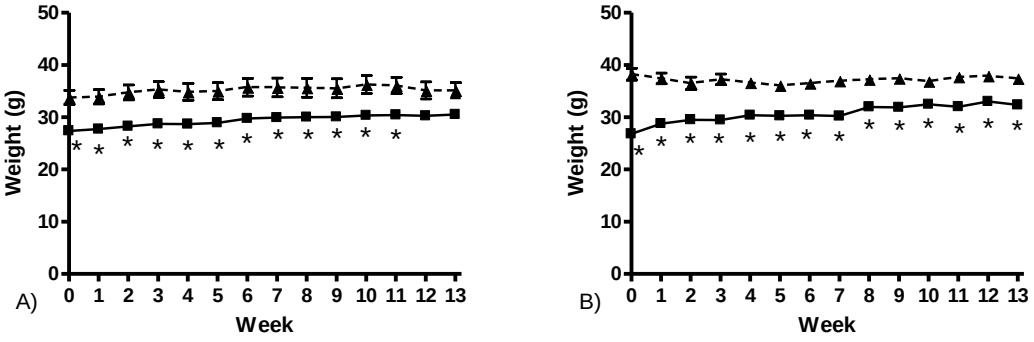


Figure 18. Effect of age on growth. Data presented as mean ± SEM. ■=Young ▲=Old A) Normal chow diet mice (n=5). B) Amino acid defined control diet mice (n=10).

Effect of SSA and Length of Treatment

Plasma. After 2 months of consumption of an amino acid defined control diet supplemented with SSA plasma 5-MTHF tended to be lower and tHcy levels tended to be

higher compared to a diet without SSA, although this was not statistically significant. Plasma 5-MTHF and tHcy levels did not differ in mice fed an amino acid defined diet containing SSA for 3 months compared to mice fed the same diet for 2 months (Table 9).

Table 9. Effect of length of treatment and SSA on plasma 5-MTHF and tHcy levels from young mice.

	Amino acid defined diets		
	2 months on diet		3 months on diet
Metabolite	CD (-SSA)	CD (+SSA)	CD (+SSA)
5-MTHF (nmol/L)	109.6 ± 39.2	78.5 ± 20.2	78.6 ± 9.2
tHcy (µmol/L)	7.4 ± 1.9	8.3 ± 2.0	10.7 ± 1.9

Values expressed as mean ± SD (n=6-8)

Effect of Age and Type of Control Diet

Plasma. Type of control diet (normal chow versus amino acid defined) did not influence many of the methylation metabolites in plasma (Table 10). Young mice fed an amino acid defined control diet had significantly lower 5-MTHF levels, which was associated with approximately a 2-fold increase in both tHcy and SAH compared to age-matched mice fed a normal chow diet. Old mice fed an amino acid defined control diet also had significantly reduced 5-MTHF levels, compared to age-matched mice fed a normal chow diet. The change in 5-MTHF in old mice fed an amino acid defined control diet was not accompanied by a change in tHcy or SAH.

Age did not impact plasma methylation metabolites in mice reared on a normal chow diet, except for cystathionine, which was decreased in old mice (Table 10). However, age impacted plasma methylation metabolites much more in mice reared on an

amino acid defined control diet. Surprisingly, old mice reared on an amino acid defined control diet had significantly lower plasma tHcy levels with no change in 5-MTHF status compared to young mice fed the same diet. Additionally, old mice fed an amino acid defined control diet had reduced levels of SAM and SAH which was associated with a 2.3-fold increase in the SAM/SAH ratio compared to young mice reared on the same diet.

Table 10. Plasma methylation metabolites: effect of age and control diet.

	YOUNG		OLD	
Metabolite	Normal Chow Diet	Amino Acid Defined Diet	Normal Chow Diet	Amino Acid Defined Diet
5-MTHF (nmol/L)	133.6 ± 53.7	78.6 ± 9.2*	107.5 ± 23.1	73.9 ± 16.8*
HCY	6.0 ± 1.8	10.7 ± 1.9*	4.3 ± 1.6	3.5 ± 1.1^
SAM	0.28 ± 0.10	0.33 ± 0.12	0.23 ± 0.04	0.20 ± 0.05^
SAH	0.07 ± 0.04	0.15 ± 0.04*	0.06 ± 0.02	0.05 ± 0.04^
SAM/SAH	5.3 ± 5.4	2.3 ± 1.0	4.4 ± 1.1	5.2 ± 3.4^
CYSTA	1.5 ± 0.6	2.3 ± 0.8	0.85 ± 0.20^	1.1 ± 0.4^
MET	87.7 ± 10.9	76.4 ± 24.3	95.6 ± 37.6	99.3 ± 39.5
BET	44.9 ± 20.2	25.7 ± 5.4	62.2 ± 35.6	36.0 ± 19.3
CHO	12.2 ± 3.3	11.1 ± 2.9	10.4 ± 3.4	13.0 ± 3.7
ADMA	0.55 ± 0.10	0.59 ± 0.06	0.62 ± 0.04	0.62 ± 0.12
SDMA	0.16 ± 0.03	0.18 ± 0.02	0.18 ± 0.01	0.19 ± 0.04

µmol/L, Values expressed as mean ± SD (n=5-8)
* $p < 0.05$ Compared to NCD of same age
^ $p < 0.05$ Compared to young of same diet

Peripheral tissues. In peripheral tissues significant changes were observed between the two control diets (Table 11). Liver tissues from young and old mice fed an amino acid defined control diet had significantly decreased SAM levels, and significantly increased ADMA and SDMA levels compared to age-matched mice fed a normal chow diet. Additionally, old mice fed an amino acid defined control diet also had elevated SAH levels leading to a reduced SAM/SAH ratio in liver tissues compared to old mice fed a normal chow diet. Kidney tissues from young mice reared on an amino acid defined control diet had significantly elevated SAM, SAH, and SDMA concentrations

compared to young mice fed a normal chow diet. In contrast, kidney tissues from old mice fed an amino acid defined diet only had elevated ADMA levels compared to age-match mice reared on a normal chow diet. Heart tissues from both young and old mice reared on an amino acid defined control diet had significantly increased SAH and SDMA levels, along with a decrease in the SAM/SAH ratio compared to age-matched mice fed a normal chow diet. Furthermore, SAM was also decreased in heart tissues from young mice reared on an amino acid defined control diet compared to young mice fed a normal chow diet.

Table 11. Peripheral tissue methylation metabolites: effect of age and control diet.

	YOUNG		OLD	
Metabolite	Normal Chow Diet	Amino Acid Defined Diet	Normal Chow Diet	Amino Acid Defined Diet
		LIVER		
SAM	63.3 ± 5.6	48.3 ± 9.2*	89.0 ± 18.9^	45.1 ± 9.1*
SAH	22.5 ± 7.9	26.5 ± 10.7	10.4 ± 2.5^	32.4 ± 9.4*
SAM/SAH	3.2 ± 1.4	2.0 ± 0.7	8.7 ± 1.5^	1.6 ± 0.7*
ADMA	0.21 ± 0.09	0.58 ± 0.08*	0.32 ± 0.28	0.86 ± 0.20^*
SDMA	0.25 ± 0.11	0.41 ± 0.07*	0.15 ± 0.07	0.60 ± 0.19^*
		KIDNEY		
SAM	24.0 ± 6.3	32.5 ± 6.0*	31.9 ± 6.2	26.3 ± 5.5
SAH	14.5 ± 1.9	26.9 ± 5.4*	23.7 ± 3.5^	26.2 ± 6.1
SAM/SAH	1.7 ± 0.5	1.23 ± 0.4	1.4 ± 0.5	1.1 ± 0.4
ADMA	0.54 ± 0.09	0.63 ± 0.09	0.64 ± 0.17	0.88 ± 0.20^*
SDMA	0.55 ± 0.13	0.76 ± 0.13*	0.73 ± 0.23	1.1 ± 0.5
		HEART		
SAM	38.2 ± 3.8	32.3 ± 4.9*	36.3 ± 4.7	34.6 ± 3.8
SAH	1.0 ± 0.1	1.6 ± 0.4*	0.63 ± 0.10^	2.3 ± 0.2^*
SAM/SAH	39.0 ± 8.8	20.3 ± 3.1*	58.4 ± 10.8^	15.2 ± 2.1^*
ADMA	1.8 ± 0.2	1.6 ± 0.2	1.9 ± 0.3	2.1 ± 0.6^
SDMA	0.21 ± 0.03	0.32 ± 0.06*	0.23 ± 0.04	0.44 ± 0.12^*

nmol/g, Values expressed as mean ± SD (n=4-8)

* $p < 0.05$ Compared to NCD of same age

^ $p < 0.05$ Compared to young of same diet

In peripheral tissues age did not affect methylation metabolites nearly as much as type of control diet (Table 11). SAM and the SAM/SAH ratio were significantly increased, whereas SAH was reduced in liver tissues from old mice fed a normal chow diet compared to young mice reared on the same diet. These same age-related effects were not observed in old mice fed an amino acid defined control diet. Liver tissues from those mice however, had increased ADMA, SDMA and betaine levels compared to young mice fed the same diet (Table 11 and Figure 19).

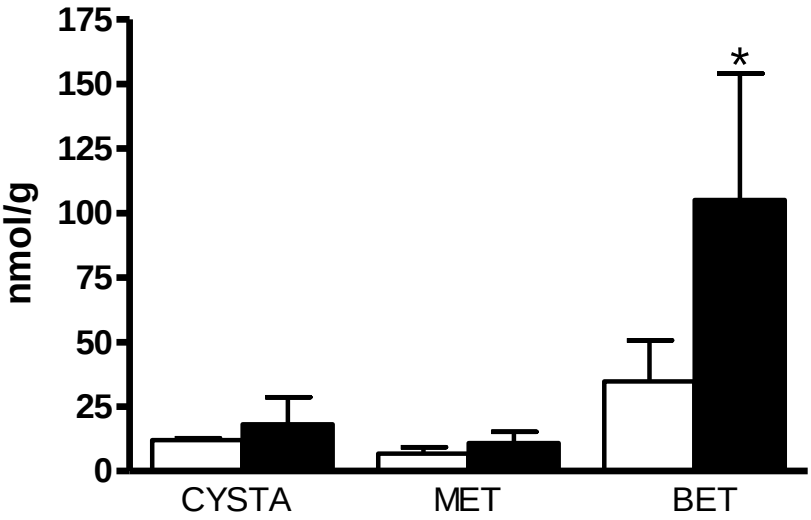


Figure 19. Liver tissue levels of cystathionine, methionine, and betaine from mice fed an amino acid defined control diet: effect of age. Data presented as mean ± SD. White bars represent young mice and black bars represent old mice. * $p < 0.05$ (n=7-8).

In kidney tissues the only methylation metabolite affected by age in the normal chow diet groups was SAH, which was increased in the old mice. Similarly, an age-related increase in ADMA in kidney tissues from mice fed an amino acid defined control diet was the only change observed in this group. However, methylation metabolites were influenced greatly by age in heart tissues. Heart tissues from old mice reared on an

amino acid defined control diet had increased SAH associated with a decreased SAM/SAH ratio along with a significant increase in ADMA and SDMA compared to young mice fed the same diet. Heart tissues from old mice reared on a normal chow diet had decreased SAH levels resulting in an increase in the SAM/SAH ratio compared to young mice fed the same diet.

In liver tissues relative mRNA levels for eight different enzymes related to the methylation cycle were evaluated in young and old mice fed an amino acid defined control diet (Figure 20). Age played a role in three out of the eight enzymes assessed, BHMT, MTR, and MTRR (methionine synthetase reductase). BHMT was significantly decreased in old mice while both, MTR and MTRR were significantly increased compared to young mice also reared on an amino acid defined control diet. The remaining five enzymes were not affected by age, however, MAT mRNA levels tended to be higher but this did not reach statistical significance ($p = 0.086$).

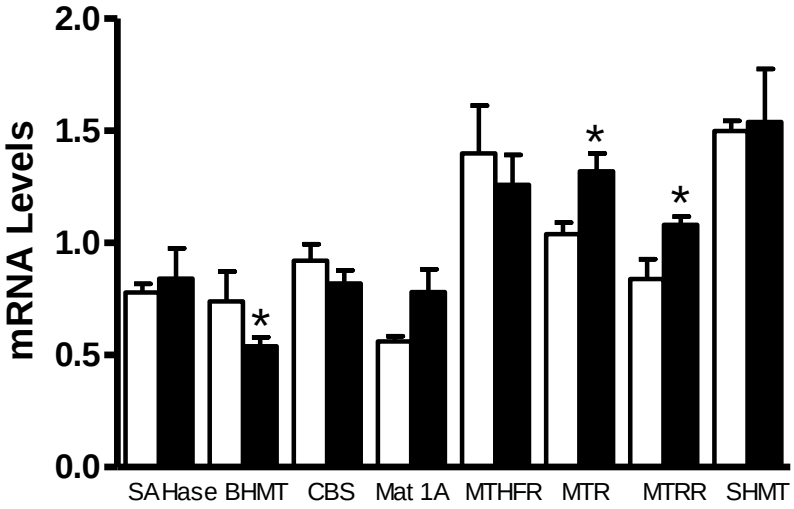


Figure 20. Liver mRNA levels of various enzymes involved in the methylation and transsulfuration pathways from mice fed an amino acid defined control diet: effect of age. Data presented as mean ± SD. White bars represent young mice and black bars represent old mice. *$p<0.05$ (n=5).

Brain tissues. The effect of a normal chow diet versus an amino acid defined control diet on CNS methylation metabolites was very similar in both young and old mice (Tables 12 and 13). Rearing on an amino acid defined control diet caused an increase in both SAM and SAH with a decrease in the SAM/SAH ratio in both age groups compared to age-matched mice fed a normal chow diet. However, the significant changes were region specific and varied slightly depending upon age group. A reduction in ADMA and SDMA was also associated with rearing on an amino acid defined control diet. Again the changes were region specific with the cortex and cerebellum being significantly affected throughout. Young mice fed an amino acid defined control diet

Table 12. Brain tissue (striatum, hippocampus, and frontal cortex) methylation metabolites: effect of age and control diet.

	YOUNG		OLD	
Metabolite	Normal Chow Diet	Amino Acid Defined Diet	Normal Chow Diet	Amino Acid Defined Diet
		STRIATUM		
SAM	12.3 ± 2.3	16.4 ± 1.8*	11.3 ± 1.8	16.5 ± 2.7*
SAH	8.7 ± 1.5	9.3 ± 0.7	6.3 ± 1.1^	9.5 ± 1.8*
SAM/SAH	1.4 ± 0.3	1.8 ± 0.3	1.8 ± 0.2^	1.7 ± 0.2
ADMA	0.37 ± 0.04	0.21 ± 0.02*	0.48 ± 0.32	0.24 ± 0.03
SDMA	0.57 ± 0.12	0.32 ± 0.07*	0.61 ± 0.19	0.46 ± 0.13^
		HIPPOCAMPUS		
SAM	16.8 ± 2.3	21.8 ± 1.9*	17.7 ± 3.7	22.0 ± 3.0
SAH	2.7 ± 0.3	3.1 ± 0.6	3.0 ± 0.5	3.0 ± 0.3
SAM/SAH	6.1 ± 0.3	7.4 ± 1.7	5.9 ± 0.3	7.3 ± 01.0*
ADMA	0.39 ± 0.07	0.29 ± 0.02*	0.43 ± 0.06	0.31 ± 0.04*
SDMA	0.51 ± 0.08	0.35 ± 0.02*	0.60 ± 0.09	0.47 ± 0.10^
		FRONTAL CORTEX		
SAM	15.1 ± 1.9	18.3 ± 2.1*	14.6 ± 2.4	18.0 ± 0.8*
SAH	2.6 ± 0.3	3.8 ± 0.9*	2.2 ± 0.4	4.0 ± 0.7*
SAM/SAH	5.9 ± 1.4	5.1 ± 1.3	6.6 ± 0.6	4.6 ± 1.0*
ADMA	0.35 ± 0.02	0.23 ± 0.05*	0.42 ± 0.11	0.47 ± 0.30^
SDMA	0.46 ± 0.05	0.28 ± 0.03*	0.49 ± 0.05	0.44 ± 0.05^

nmol/g, Values expressed as mean ± SD (n=4-8)

* $p < 0.05$ Compared to NCD of same age

^ $p < 0.05$ Compared to young of same diet

also had an increase in cortical cystathionine levels compared to young normal chow diet mice.

Table 13. Brain tissue (cortex, cerebellum, and mid-brain) methylation metabolites: effect of age and control diet.

	YOUNG		OLD	
Metabolite	Normal Chow Diet	Amino Acid Defined Diet	Normal Chow Diet	Amino Acid Defined Diet
		CORTEX		
SAM	16.9 ± 0.8	16.5 ± 1.5	15.8 ± 1.2	16.5 ± 1.3
SAH	2.2 ± 0.2	3.3 ± 0.4*	1.9 ± 0.2	3.6 ± 0.4*
SAM/SAH	8.0 ± 0.8	5.0 ± 0.4*	8.2 ± 0.5	4.7 ± 0.7*
CYSTA	16.5 ± 3.8	23.1 ± 2.6*	30.5 ± 6.4^	30.6 ± 5.0^
MET	64.2 ± 11.0	62.6 ± 8.0	58.9 ± 8.3	63.3 ± 13.3
BET	12.4 ± 5.2	12.5 ± 4.5	17.3 ± 6.6	14.9 ± 4.8
ADMA	0.25 ± 0.02	0.19 ± 0.01*	0.26 ± 0.02	0.22 ± 0.03^*
SDMA	0.38 ± 0.05	0.26 ± 0.02*	0.45 ± 0.08	0.36 ± 0.04^*
		CEREBELLUM		
SAM	20.2 ± 2.6	21.5 ± 1.5	18.8 ± 2.0	20.9 ± 4.5
SAH	2.0 ± 0.2	2.9 ± 0.3*	1.8 ± 0.3	3.0 ± 0.9*
SAM/SAH	10.1 ± 0.8	7.5 ± 1.0*	10.8 ± 1.3	6.9 ± 1.3*
CYSTA	n/a	92.0 ± 12.1	n/a	81.0 ± 18.8
MET	n/a	80.6 ± 16.3	n/a	71.9 ± 18.4
BET	n/a	34.5 ± 6.8	n/a	36.8 ± 6.3
ADMA	0.31 ± 0.04	0.20 ± 0.01*	0.33 ± 0.04	0.23 ± 0.08*
SDMA	0.40 ± 0.09	0.24 ± 0.02*	0.47 ± 0.10	0.34 ± 0.08^*
		MID-BRAIN		
SAM	19.0 ± 1.0	18.1 ± 5.7	17.9 ± 1.8	14.8 ± 3.6
SAH	2.07 ± 0.29	3.62 ± 0.88*	1.86 ± 0.32	3.23 ± 0.61*
SAM/SAH	9.30 ± 1.15	4.94 ± 0.70*	9.82 ± 1.48	4.55 ± 0.57*
CYSTA	32.8 ± 3.9	41.8 ± 15.7	48.4 ± 11.1^	51.1 ± 19.8
MET	52.4 ± 6.2	52.7 ± 19.5	55.9 ± 14.3	48.8 ± 16.8
BET	21.0 ± 6.9	22.6 ± 9.8	28.7 ± 10.1	23.8 ± 8.8
ADMA	0.19 ± 0.01	0.21 ± 0.03	0.20 ± 0.02	0.21 ± 0.03
SDMA	0.41 ± 0.08	0.36 ± 0.08	0.50 ± 0.12	0.46 ± 0.13

nmol/g, Values expressed as mean ± SD (n=5-8)
n/a = not avaliable
* $p < 0.05$ Compared to NCD of same age
^ $p < 0.05$ Compared to young of same diet

There were minimal effects of age on brain tissue methylation metabolites from mice fed a normal chow diet and from mice fed an amino acid defined control diet

(Tables 12 and 13). No age-related changes were observed for methionine, SAM, or betaine in either of the control diet groups. Old mice fed a normal chow diet had significantly lower striatal SAH concentrations leading to an increase in the SAM/SAH ratio compared to young mice also fed a normal chow diet. Cortical and mid-brain levels of cystathionine were also elevated due to an increase in age in mice fed a normal chow diet. Old mice fed an amino acid defined control diet also had an increase in cortical cystathionine. Additionally, ADMA (2 regions) and SDMA (5 regions) levels were significantly increased in old mice fed an amino acid defined control diet compared to young mice fed the same diet. Table 14 summarizes the effects of age and diet on the methylation metabolites analyzed in the various brain regions.

Table 14. Brain tissue methylation metabolite summary: effect of age and control diet

	The Effect of Age	
Metabolite	Normal Chow Diet	Amino Acid Defined Diet
SAM	none	none
SAH	Old: STR ↓	none
SAM/SAH	Old: STR ↑	none
CYSTA	Old: CX,MB ↑	Old: CX ↑
MET	none	none
BET	none	none
ADMA	none	Old: FCX,CX ↑
SDMA	none	Old: STR,HIP,FCX,CX,CB ↑
	The Effect of Diet	
Metabolite	Young	Old
SAM	AADD: STR,HIP,FCX ↑	AADD: STR,FCX ↑
SAH	AADD: FCX,CX ,CB,MB ↑	AADD: STR,FCX,CX,CB ,MB ↑
SAM/SAH	AADD: CX ,CB,MB↓	AADD: HIP ↑; FCX,CX,CB,MB↓
CYSTA	AADD: CX ↑	none
MET	none	none
BET	none	none
ADMA	AADD: STR,HIP,FCX,CX,CB ↓	AADD: HIP,CX,CB ↓
SDMA	AADD: STR,HIP,FCX,CX,CB ↓	AADD: CX,CB↓

Discussion

Effect of Control Diet

Young mice reared on an amino acid defined control diet for 3 months had significantly reduced plasma 5-MTHF levels and elevated tHcy levels compared to young mice reared on a normal chow diet (Table 10). Old mice reared on an amino acid defined control diet for 3 months also had significantly reduced plasma 5-MTHF levels but no change in tHcy compared to old mice reared on a normal chow diet (Table 10). The reason why plasma 5-MTHF is lower in mice reared on an amino acid defined control diet is not completely understood since the folate content is higher in the amino acid defined control diet compared to the normal chow diet. A possible explanation could be that the amino acid defined diet is supplemented with SSA, which inhibits bacterial folate synthesis in the gut, hence lowering folate levels. After 2 months of treatment 5-MTHF levels were lower as a result of the addition of SSA but this was not statistically significant (Table 9). The effect of SSA after 3 months of treatment was not assessed in this dissertation, thus it is possible that during a longer treatment period statistically significant differences in plasma 5-MTHF levels would develop between diets with and without SSA. This idea is supported by Kim and co-workers, who reported that in 3 week old rats treated with amino acid defined diets with and without SSA for 4 weeks resulted in different 5-MTHF levels, the diet containing SSA, had lower 5-MTHF levels (Kim et al. 1994). Although in the aforementioned study treatment length was shorter than our 2 month study, the discrepancy in 5-MTHF results might be due to the fact that they used very young rats. Furthermore, variation in absorption of nutrients between the normal chow and amino acid defined control diets may account for the difference in

plasma 5-MTHF. In young mice fed an amino acid defined control diet the increase in tHcy can be attributed to the decrease in 5-MTHF. However, in old mice this was not the case. There are additional factors that may influence tHcy levels, thus in old mice some factor(s) may account for the lower level of plasma tHcy associated with reduced plasma 5-MTHF.

It is not clear why plasma SAH and tHcy are elevated in young mice fed an amino acid defined diets compared to mice fed a normal chow diet (Table 10). Liver tissue has a high demand for methyl groups and is the most active organ for methylation reactions. The majority of plasma tHcy and SAH is derived from methylation reactions originating in the liver, although the plasma SAM/SAH ratio was not significantly different between normal chow and amino acid defined diets in young mice (Table 10). However, the SAM/SAH ratio is significantly reduced in the heart of young and old mice and in the liver of old mice fed an amino acid defined control diet (Table 11). In addition, the SAM/SAH ratio was also reduced in the liver of old mice fed an amino acid defined control diet (Table 11). Presumably other dietary factors may exert a tissue specific influence on methylation reactions depending on the activities of methyltransferases in different cells. The type of control diet also affected ADMA and SDMA levels in the peripheral tissues in both young and old mice (Table 11). Generally, higher concentrations of these metabolites were found in mice reared on amino acid defined diets. These findings may have important implication for investigators studying the effect of HHcy on ADMA concentrations, specifically with respect to vascular disease. Higher ADMA levels inhibit endothelial function, and the type of diet fed to mice should be taken into consideration.

Generally, in brain tissue SAM and SAH levels were elevated and the SAM/SAH ratio decreased, thus SAH was the driving factor of the SAM/SAH ratio in both young and old mice reared on an amino acid defined diet compared to those fed a normal chow diet, these changes were region specific (Tables 12 and 13). Although these differences are not easy to reconcile they do suggest that care should be taken in comparing methylation metabolite data from laboratories using different diets. Folic acid was not the only compound altered between the two types of control diet. Vitamin B_{12} and choline levels were lower in the amino acid defined control diet, whereas vitamin B_6 was higher. However, these nutritional differences do not appear to be responsible for the discrepancies in the methylation metabolite concentrations between the two control diets.

Young and old mice fed an amino acid defined control diet had reduced ADMA and SDMA levels in several brain regions compared to mice fed a normal chow diet (Tables 12 and 13). It is important to note an amino acid defined control diet appears to influence brain and peripheral tissues differently, and tissue levels do not correlate with plasma levels. Most studies to date that have investigated the effects of HHcy on peripheral and cerebral vascular function have measured plasma ADMA levels (Guldiken et al. 2007; Dayla and Lentz 2005; Stuhlinger et al. 2003). The data shown here underscores the importance of measuring tissue metabolite levels rather than relying on plasma levels. Interestingly, this is the only investigation that has analyzed methylation metabolite differences between a normal chow diet and amino acid defined control diet.

Effect of Age

This dissertation reports no significant age-related differences between plasma 5-MTHF levels in mice reared on an amino acid defined control diet (Table 10). However,

there was a significant age-related decline in plasma tHcy (Table 10). Plasma 5-MTHF status was previously reported in young and old mice by Keyes and co-workers in 2007; however, no age effects were found (Keyes et al. 2007). Unfortunately, the laboratory did not report tHcy levels in the 4 and 18 month old mice. In another study, 14 month old *tg*-MTHFR wild type mice had plasma tHcy levels of approximately 5 µmol/L, similar to our results of 3.5 µmol/L in 23 month old C57BL/6J mice (Knock et al. 2008). Their report did not include plasma tHcy levels in young *tg*-MTHFR mice. The age-related decline in tHcy levels presented in this chapter appears not to be caused by folate status but rather to other factor(s) that can influence tHcy levels. This observation was unexpected since in humans plasma tHcy levels increase with age (Selhub et al. 1993; Nilsson et al. 1994; Carmel et al. 1999; Bottiglieri et al. 2000). However, plasma tHcy levels may decrease with age in mice due to increased enzymatic activity of MTR and MTRR two enzymes involved in the remethylation of Hcy to methionine. This theory is supported by the mRNA data in liver tissues of aged mice since liver is presumed to be the main contributor of plasma tHcy (Refsum et al. 1998), total mRNA was increased for MTR and MTRR in old mice compared to young mice (Figure 20).

In addition, there was an age-related decrease in SAM and SAH, and an increase in the SAM/SAH ratio in plasma, thus again SAH was the metabolite that had the most influence on the methylation ratio (Table 10). This contrasted with the results found in peripheral tissues which showed no major age-related differences in SAM, SAH or the ratio, with the exception of heart tissue which had an elevation in SAH and consequently a decreased SAM/SAH ratio (Table 11). To our knowledge this is the first study describing age-related changes in SAM and SAH in peripheral tissues of mice. Betaine

was elevated approximately 3-fold in the liver tissue from old mice fed an amino acid defined diet possibly due to decreased BHMT activity as evidenced from reduced BHMT mRNA levels in the liver of old mice (Figures 19 and 20). As previously mentioned liver mRNA levels of MTR and MTRR were increased in old mice, this could potentially be associated with either reduced or increased enzyme activity. Generally, increased mRNA results in increased enzyme activity due to higher levels of enzyme present (Bera et al. 2008; Huang et al. 2010). However, it has been reported that increased mRNA expression is a compensation response to decreased enzymatic activity (van Boxel-Dezaire et al. 1999). Additionally, enzymatic activity can be altered without any change in mRNA levels (Yamada et al. 2000). If associated with increased activity then these results contrast with reports by Finkelstein and Mays, who showed that MTR activity is down in the liver of old rats and that CBS activity is up-regulated, suggesting a redirection of Hcy away from the methylation pathway and towards the transsulfuration pathway (Finkelstein et al. 1975; Mays et al. 1973). In the present study cystathionine levels were not affected by age in the liver, lending more support to the fact that the transsulfuration pathway may not be upregulated in old mice. However, cystathionine γ-lyase is present in liver, hence, accumulation of cystathionine may not occur, but rather an accumulation of cysteine.

An interesting observation is the age-related increase in ADMA and SDMA in peripheral tissues, which has never been reported (Table 11). Elevated concentrations of these metabolites could be due to an increased rate of methylation of arginine residues in proteins, or increased proteolysis of methylated arginine residues giving rise to free ADMA and SDMA. Additionally, older mice may have an impairment in renal function,

thereby decreasing the rate of elimination from the body. Regardless of the mechanism(s) involved increased tissue levels of ADMA can lead to inhibition of eNOS and endothelial dysfunction in aged mice (Guldiken et al. 2007; Dayal and Lentz 2005; Stuhlinger et al. 2003).

There were no changes in SAM, SAH, or the SAM/SAH ratio in any of the brain regions from old mice fed the amino acid defined control diet compared to young fed the same diet (Tables 12 and 13). This data conflicts with a report describing age-related differences in SAM and SAH in brain tissues from wild type APP (amyloid precursor protein)/PS1 mice (transgenic AD model) (Hooijman and Henk 2009). The study reported decreased levels of SAM and SAH, with no change in the SAM/SAH ratio in the frontal brain tissue of 15 month old mice compared to 8 month old mice. Age-related decline of theses metabolites has also previously been reported in rats (Stramentinoli et al. 1977). The discrepancies between this study and the previous studies may be due to differences in the ages of the rodents studied, strain background, brain region analyzed, or due impart that the old mice evaluated in this study had low levels of plasma tHcy compared to young mice. Plasma tHcy levels were not evaluated in the previously mentioned studies. Another interesting finding is that cystathionine was significantly elevated in the cortex of old compared to young mice fed an amino acid defined control diet (Table 13). This observation suggests that the transsulfuration pathway may be upregulated in brain tissues with aging. This young and old study was from mice sacrificed by CO_2 asphyxiation, it is possible that age-related methylation differences would be observed when sacrificed by microwave radiation, a method that inhibits all post-mortem effects (discussed further in chapter 4).

Elevations of ADMA and SDMA were also present in the brain of old mice fed an amino acid defined diet (Tables 12 and 13). Again, elevated concentrations of these metabolites could be due to an increased rate of methylation of arginine residues in proteins, or increased proteolysis of methylated arginine residues giving rise to free ADMA and SDMA. Reduced renal clearance could also be implicated in this elevation.

Effect of Length of Treatment

Length of treatment on an amino acid defined control diet, 2 versus 3 months, was evaluated in young mice and did not cause any significant differences in 5-MTHF or tHcy levels (Table 9). In 2003, Delvin reported that mice reared on a folate deficient diet for 7 or 15 weeks did not alter plasma tHcy (Delvin et al. 2004). This can be explained by a study that found that 5-MTHF levels equilibrate after 5 weeks of treatment, thus remaining on the treatment longer does not induce a more deficient state (Lemon et al. 2008). In this study, we chose to treat for 3 or 6 months with an amino acid defined diet containing SSA as the control diet for the folate deficient studies.

Summary

This is the first study reporting altered ADMA and SDMA concentrations in peripheral and brain tissues of aged mice. In addition, there are only two published reports that have investigated methylation metabolites in old mice or rats, both of which have reported SAM and SAH decrease with age, interestingly; these studies did not include any measures of plasma tHcy or 5-MTHF. In this study we were unable to confirm any age-related differences in SAM and SAH in brain tissues from mice. The most novel finding in this chapter was the age-related decline in plasma tHcy. After

extensive evaluation of length of treatment and type of control diet in regard to methylation metabolites, as shown in this chapter, it was decided that an amino acid defined control diet containing SSA would be used as the control diet in all future studies. The following chapter will assess the effect of folate status on methylation metabolites in young and old C57BL/6J mice and old *tg*-MTHFR mice.

CHAPTER FOUR

The Effect of Folate Deficiency on Methylation Metabolites in Mice Sacrificed by CO_2 Asphyxiation and Microwave Radiation.

Introduction

Folate, specifically, 5-MTHF, is a cofactor in the synthesis of methionine from Hcy, thus when folate levels are reduced HHcy can occur. Increased plasma tHcy due to genetic defects and/or nutritional deficiencies is associated with cardiovascular disease, cancers, birth defects, CNS demyelination, depression, age-related cognitive decline and AD (Clarke et al. 1998; Christensen et al. 1997; Eskes 1998; Regland et al. 1990; Baig et al. 1998; Surtees et al. 1991; Arinami et al. 1997; Bottiglieri et al. 2000). It is not understood whether elevated Hcy is directly involved in the pathologic processes or a secondary marker related to another mechanism.

Mouse models of folate deficiency either alone or in combination with vitamin deficiencies and/or other nutritional components have been used to study the effects of HHcy, the majority of which have focused on vascular function. Few studies have examined the effects of folate deficiency induced by a diet solely lacking folic acid on CNS function and brain neurochemistry. In this folate deficient study there are a total of three amino acid defined treatment diets: control diet, low folate diet, and folate deficient diet. In the previous chapter data was presented showing that length of treatment and supplementation with SSA in an amino acid defined control diet did not result in any significant differences in plasma tHcy or 5-MTHF. From these previous findings, a treatment period of 3 months on diets including SSA was selected for these studies.

One-carbon metabolism is highly regulated; consequently folate deficiency leads to significant changes in the levels of methionine, SAM, and SAH. The effect of folate deficiency on metabolites of the methylation cycle has been studied by many investigators; however, this has primarily focused on peripheral tissues and not CNS tissues. Furthermore, there are few reports that have studied the effect of folate deficiency on methylation cycle metabolites (SAM and SAH) and transsulfuration metabolites (cystathionine) in CNS tissue from aged C57BL/6J and *tg*-MTHFR mice. Similarly, there are few reports in the literature that have studied choline and betaine concentrations in CNS tissues.

The studies presented in this chapter sought to characterize the effects of folate deficiency on methylation metabolites in peripheral and CNS tissues from both young and old mice. A comprehensive analysis of metabolites associated with the methylation pathway, along with a single marker of the transsulfuration pathway was performed. In addition, levels of ADMA and SDMA were also investigated in various peripheral tissues and regional areas of brain tissue. Concentrations of plasma ADMA and SDMA have previously been shown to be elevated in mice and humans with HHcy, however, brain tissue levels have never been reported (Guldiken et al. 2007; Dayal et al. 2005; Stuhlinger et al. 2003). The studies outlined above were performed in mice that were sacrificed by CO_2 asphyxiation and by microwave radiation. To date no one has conducted an analysis of methylation cycle metabolites using microwave radiation, an extremely rapid method of sacrificing mice that eliminates any post-mortem effects.

Typically, earlier studies using microwave radiation have used internal brain temperature (>85°C) following microwave radiation to verify that the technique was

effective (Delaney and Geiger 1996; Groppetti et al. 1997; Moroji et al. 1977). However, insertion of a thermometer into the brain is not ideal for investigations that require dissection of regional brain tissues. It has been demonstrated the adenine nucleotides are rapidly converted to adenosine via nucleotidase and phosphodiesterase in under a second (Dunwiddie et al. 1997). Additionally, in the heart, hypoxia, which occurs in mice sacrificed by CO_2 asphyxiation, has been shown to inhibit adenosine kinase activity (Decking et al. 1997), which generates large amounts of adenosine. A similar mechanism is thought to occur also in brain tissues due to an increase in adenosine levels and a clearance in ATP levels following sacrifice by CO_2 asphyxiation (Schmidt et al. 1971; Sanders et al. 1984; Wu and Phillis 1978; Guidotti et al. 1974). It has also been shown that there is an inverse correlation between adenosine concentrations and microwave power setting even when internal brain temperature is >85°C (Delaney and Geiger 1996). For the aforementioned reasons, a low adenosine level (<2 nmol/g), was chosen be to the marker to identify the effectiveness of microwave radiation.

Results

C57BL/6J Aged Folate Deficient Study

Effect of microwave radiation in amino acid defined control diet mice. All mice sacrificed by microwave radiation that had adenosine levels 3 SDs from controls were omitted from analysis. In this study young mice sacrificed by CO_2 asphyxiation had adenosine levels 700-fold higher than mice sacrificed by microwave radiation (Figure 21). However, old mice sacrificed by microwave radiation had significantly higher adenosine levels (> 300 nmol/g) compared to young mice (< 2 nmol/g) also sacrificed by

microwave radiation in all three brain regions measured (Figure 22). Additionally, choline levels, which have also been shown to be sensitive to microwave radiation, were drastically different in young and old mice sacrificed by microwave radiation (data not shown). These results suggest that the microwave radiation technique worked efficiently and as expected in young mice but did not work properly in old mice. Therefore, the data from the old mice sacrificed by microwave radiation cannot be considered accurate and hence, was not analyzed for differences between the two sacrifice methods.

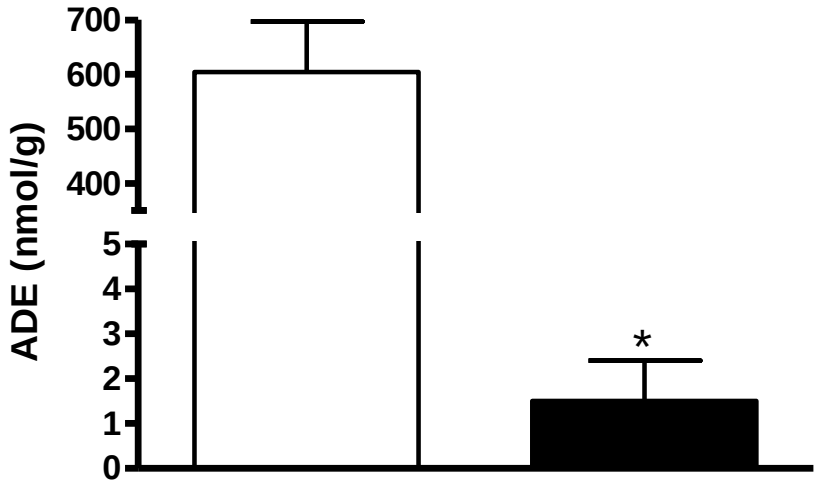


Figure 21. Striatum brain levels of adenosine from young mice fed an amino acid defined control diet. Data presented as mean ± SD. White bars represent CO_2 asphyxiated mice and black bars represent microwaved mice. * $p < 0.0001$ (n=5-6).

Unfortunately, a more comprehensive methylation metabolite analysis method was developed after all other analytic analysis had been performed. This resulted in an insufficient PCA extract volume for the comprehensive methylation metabolite testing in the striatum, hippocampus, and frontal cortex, thus not all of the methylation metabolites were measured in these regions.

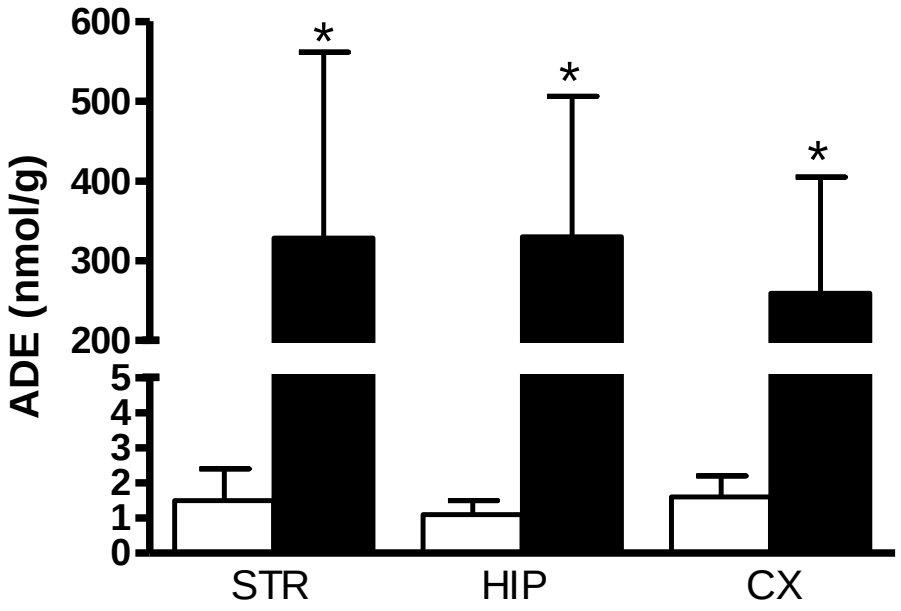


Figure 22. Regional brain levels of adenosine from mice fed an amino acid defined control diet sacrificed by microwave radiation. Data presented as mean ± SD. White bars represent young mice and black bars represent old mice. * $p < 0.01$ (n=6).

Many of the methylation metabolites investigated showed variations among the two sacrificing techniques in brain tissues from young mice (Tables 15 and 16). Microwave radiation caused a significant decrease in SAH in all brain regions; SAM was also decreased in four brain regions (striatum, hippocampus, frontal cortex and cerebellum). The SAM/SAH ratio was 4- to 25-fold higher in mice sacrificed by microwave radiation due to a decrease in SAH. Choline levels were at least 4-fold lower from mice sacrificed by microwave radiation. Betaine was significantly increased approximately 2-fold in the cortex and cerebellum from young mice sacrificed by microwave radiation compared to mice sacrificed by CO_2 asphyxiation. Cystathionine was elevated in the cortex of young microwaved mice compared to mice sacrificed by CO_2 asphyxiation. A microwave dependent increase in ADMA was present in the

hippocampus, cortex, cerebellum, and mid-brain. Similarly, SDMA was also increased in the striatum, hippocampus, frontal cortex, cortex, and cerebellum compared to mice sacrificed by CO_2 asphyxiation.

Table 15. Brain tissue (striatum, hippocampus, and frontal cortex) methylation metabolites from young mice: effect of microwave radiation.

	Amino acid defined diet	
Metabolite	CO_2 Control Diet	MW Control Diet
	STRIATUM	
SAM	16.4 ± 1.8	12.2 ± 2.3*
SAH	9.3 ± 0.7	0.33 ± 0.15*
SAM/SAH	1.8 ± 0.3	44.5 ± 22.8*
ADE	n/a	1.5 ± 0.9
ADMA	0.21 ± 0.02	0.22 ± 0.02
SDMA	0.32 ± 0.07	0.42 ± 0.05*
	HIPPOCAMPUS	
SAM	21.8 ± 1.9	8.4 ± 2.7*
SAH	3.1 ± 0.6	0.31 ± 0.11*
SAM/SAH	7.4 ± 1.7	31.7 ± 16.4 *
ADE	n/a	1.1 ± 0.4
ADMA	0.29 ± 0.02	0.36 ± 0.04*
SDMA	0.35 ± 0.02	0.24 ± 0.06*
	FRONTAL CORTEX	
SAM	18.3 ± 2.1	15.1 ± 1.0*
SAH	3.8 ± 0.9	0.21 ± 0.07*
SAM/SAH	5.1 ± 1.3	78.3 ± 24.5*
ADE	n/a	1.3 ± 0.5
ADMA	0.23 ± 0.05	0.24 ± 0.04
SDMA	0.28 ± 0.03	0.34 ± 0.05*

nmol/g, Values expressed as mean ± SD (n=6-8)
n/a = not avaliable
* $p < 0.05$ Compared to CO_2 of same region

Effect of folate deficiency on weight. The amino acid defined low folate diet did not influence weight; however the diet lacking folate completely (folate deficient) did decrease weight compared to control diet mice of the same age (Figure 23). Young and old mice fed the folate deficient diet began to lose weight after 9 weeks of treatment

Table 16. Brain tissue (cortex, cerebellum, and mid-brain) methylation metabolites from young mice: effect microwave radiation

	Amino acid defined diet	
Metabolite	CO_2 Control Diet	MW Control Diet
	CORTEX	
SAM	16.5 ± 1.5	14.3 ± 4.6
SAH	3.3 ± 0.4	0.28 ± 0.17*
SAM/SAH	5.0 ± 0.4	65.2 ± 33.2*
ADE	n/a	1.6 ± 0.6
CYSTA	23.1 ± 2.6	36.1 ± 7.4*
MET	62.6 ± 8.0	70.2 ± 11.6
BET	12.5 ± 4.5	30.1 ± 6.8*
CHO	165.4 ± 28.3	30.8 ± 6.7*
ADMA	0.19 ± 0.01	0.38 ± 0.03*
SDMA	0.26 ± 0.02	0.38 ± 0.07*
	CEREBELLUM	
SAM	21.5 ± 1.5	18.1 ± 3.2*
SAH	2.9 ± 0.3	0.33 ± 0.04*
SAM/SAH	7.5 ± 1.0	54.9 ± 9.5*
ADE	n/a	2.0 ± 1.1
CYSTA	92.0 ± 12.1	96.8 ± 11.1
MET	80.6 ± 16.3	69.7 ± 6.0
BET	34.5 ± 6.8	57.8 ± 5.5*
CHO	129.3 ± 24.8	34.7 ± 12.2*
ADMA	0.20 ± 0.01	0.27 ± 0.03*
SDMA	0.24 ± 0.02	0.30 ± 0.06*
	MID-BRAIN	
SAM	18.1 ± 5.7	12.4 ± 3.5
SAH	3.6 ± 0.9	0.28 ± 0.03*
SAM/SAH	4.9 ± 0.7	44.1 ± 12.7*
ADE	n/a	1.3 ± 0.6
CYSTA	41.8 ± 15.7	57.5 ± 7.4
MET	52.7 ± 19.5	44.1 ± 12.7
BET	22.6 ± 9.8	42.0 ± 10.0
CHO	299.1 ± 146.2	33.6 ± 58*
ADMA	0.21 ± 0.03	0.14 ± 0.01*
SDMA	0.36 ± 0.08	0.35 ± 0.06

nmol/g, Values expressed as mean ± SD (n=6-8)

n/a = not avaliable

* $p < 0.05$ Compared to CO_2 of same region

this was statistically significant at 10 weeks for young mice and 11 weeks for old mice. Both groups continued to decline in weight until the end of treatment.

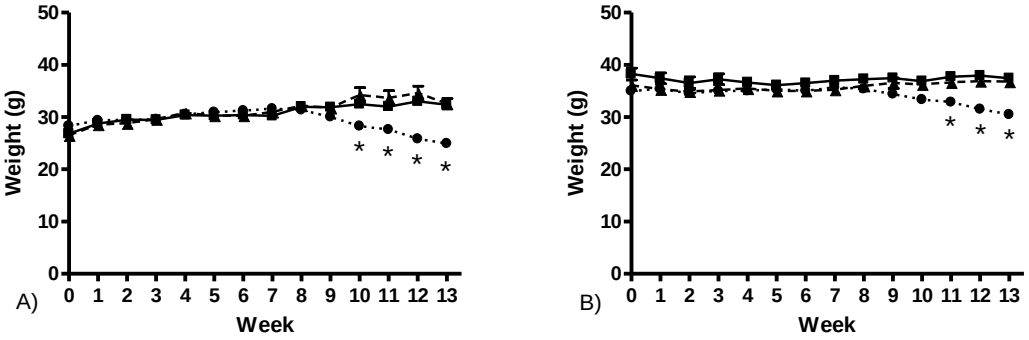


Figure 23. Effect of folate on growth. Data presented as mean ± SEM. ■=CD, ▲=LF, ●=FD. A) Young mice (n=8-14). B) Old mice (n=10).

Effect of folate deficiency in young and old mice sacrificed by CO_2 asphyxiation. The effect of folate deficiency on plasma methylation metabolites was very similar for both old and young C57BL/6J mice (Tables 17 and 18). Young and old mice fed low and folate deplete diets had a significant decrease in plasma 5-MTHF with a substantial increase in tHcy compared to age-matched mice fed control diet. There is an inverse relationship between 5-MTHF and tHcy. Old mice fed a folate deficient diet appeared to be the most effected having the lowest 5-MTHF and highest tHcy levels of any group. Young mice reared on a folate deficient diet had a decrease in plasma SAM associated with an increase in SAH, thus, the SAM/SAH ratio was significantly decreased compared to mice fed control diet. Furthermore, young mice on a low folate diet also had a significant reduction in the SAM/SAH ratio, although changes in SAM and SAH were not significant. Old mice reared on a folate deficient diet had a decreased SAM/SAH

ratio in plasma, which was influenced only by a decrease in SAH, with no change in SAM present compared to old mice fed a control diet.

Table 17. Plasma methylation metabolites from young mice sacrificed by CO_2 asphyxiation: effect of folate.

	YOUNG		
Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
5-MTHF (nmol/L)	78.6 ± 9.2	12.1 ± 4.9*	6.8 ± 6.0*
HCY	10.7 ± 1.9	24.1 ± 7.1*	72.9 ± 20.0*
SAM	0.33 ± 0.12	0.26 ± 0.04	0.19 ± 0.03*
SAH	0.15 ± 0.04	0.26 ± 0.07	0.42 ± 0.13*
SAM/SAH	2.3 ± 1.0	1.1 ± 0.4*	0.49 ± 0.14*
CYSTA	2.3 ± 0.8	3.5 ± 1.2	5.1 ± 2.9
MET	76.4 ± 24.3	46.6 ± 32.7	83.0 ± 29.7
BET	25.7 ± 5.4	26.0 ± 15.1	35.6 ± 17.9
CHO	11.1 ± 2.9	9.5 ± 3.7	7.9 ± 1.8
ADMA	0.59 ± 0.06	0.61 ± 0.08	0.55 ± 0.14
SDMA	0.18 ± 0.02	0.19 ± 0.03	0.21 ± 0.05

µmol/L, Values expressed as mean ± SD (CD & LF n=8; FD n=15-16)
* $p < 0.05$ Compared to CD

Table 18. Plasma methylation metabolites from old mice sacrificed by CO_2 asphyxiation: effect of folate.

	OLD		
Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
5-MTHF (nmol/L)	73.9 ± 16.8	9.2 ± 5.6*	3.53 ± 1.49*
HCY	3.5 ± 1.1	13.7 ± 2.4*	95.6 ± 30.6*
SAM	0.20 ± 0.05	0.19 ± 0.05	0.26 ± 0.09
SAH	0.05 ± 0.04	0.09 ± 0.06	0.95 ± 0.56*
SAM/SAH	5.2 ± 3.4	3.1 ± 1.9	0.43 ± 0.38*
CYSTA	1.1 ± 0.4	5.9 ± 7.5	4.9 ± 3.2
MET	99.3 ± 39.5	116.7 ± 77.0	95.8 ± 28.5
BET	36.0 ± 19.3	16.8 ± 3.0	30.6 ± 16.8
CHO	13.0 ± 3.7	8.6 ± 1.5	10.9 ± 3.5
ADMA	0.62 ± 0.12	0.55 ± 0.18	0.68 ± 0.17
SDMA	0.19 ± 0.04	0.17 ± 0.04	0.20 ± 0.04

µmol/L, Values expressed as mean ± SD (n=5-7)
* $p < 0.05$ Compared to CD

The effects of folate deficiency in the peripheral tissues from young and old mice are reported in tables 19 and 20, respectively. Mice fed a low folate diet had reduced SAM levels in liver tissues resulting in a decreased SAM/SAH ratio. Moreover, young and old mice reared on a folate deficient diet had severely diminished SAM levels with a contaminant increase in SAH resulting in a reduced SAM/SAH ratio in liver tissues. In addition in liver tissues, folate deficiency depleted betaine levels in both age groups. Increased liver tissue levels of ADMA and SDMA were observed in young mice fed a folate deficient diet compared to mice fed a control diet.

Table 19. Peripheral tissue methylation metabolites from young mice sacrificed by CO_2 asphyxiation: effect of folate.

	YOUNG		
Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
		LIVER	
SAM	48.3 ± 9.2	23.2 ± 6.5*	11.5 ± 2.6*
SAH	26.5 ± 10.7	37.1 ± 10.5	82.7 ± 15.4*
SAM/SAH	2.0 ± 0.7	0.70 ± 0.33*	0.14 ± 0.03*
CYSTA	12.1 ± 3.1	13.2 ± 8.0	16.4 ± 8.6
MET	6.8 ± 2.4	4.4 ± 1.7	7.8 ± 3.8
BET	34.8 ± 15.9	27.4 ± 15.0	13.4 ± 3.7*
ADMA	0.58 ± 0.08	0.56 ± 0.06	0.94 ± 0.17*
SDMA	0.41 ± 0.07	0.43 ± 0.08	0.65 ± 0.16*
		KIDNEY	
SAM	32.5 ± 6.0	29.5 ± 7.1	27.9 ± 4.7
SAH	26 ± 5.4	24.7 ± 7.1	27.5 ± 4.3
SAM/SAH	1.3 ± 0.4	1.3 ± 0.6	1.1 ± 0.3
ADMA	0.63 ± 0.09	0.66 ± 0.14	0.81 ± 0.15*
SDMA	0.76 ± 0.13	0.80 ± 0.17	0.74 ± 0.11
		HEART	
SAM	32.3 ± 4.9	34.0 ± 3.0	29.8 ± 3.7
SAH	1.6 ± 0.4	3.9 ± 1.8	11.7 ± 4.1*
SAM/SAH	20.3 ± 3.1	10.2 ± 4.1*	2.8 ± 1.0*
ADMA	1.6 ± 0.2	1.9 ± 0.3*	1.7 ± 0.3
SDMA	0.32 ± 0.06	0.38 ± 0.06	0.35 ± 0.07

nmol/g, Values expressed as mean ± SD (CD & LF n=8; FD n=15-16)
* $p < 0.05$ Compared to CD of same region

Table 20. Peripheral tissue methylation metabolites from old mice sacrificed by CO_2 asphyxiation: effect of folate.

	OLD		
Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
		LIVER	
SAM	45.1 ± 9.1	22.2 ± 5.9*	12.0 ± 5.58*
SAH	32.4 ± 9.4	43.4 ± 4.3	62.6 ± 14.9*
SAM/SAH	1.6 ± 0.7	0.51 ± 0.14*	0.20 ± 0.12*
CYSTA	18.1 ± 10.5	24.4 ± 12.4	22.4 ± 18.3
MET	10.8 ± 4.5	10.6 ± 3.6	14.8 ± 8.5
BET	105.0 ± 49.1	28.0 ± 4.8*	13.1 ± 6.5*
ADMA	0.86 ± 0.20	0.77 ± 0.14	0.85 ± 0.48
SDMA	0.60 ± 0.19	0.46 ± 0.11	0.43 ± 0.09
		KIDNEY	
SAM	26.3 ± 5.5	20.2 ± 8.5	29.2 ± 6.0
SAH	26.2 ± 6.1	22.1 ± 9.9	31.9 ± 11.2
SAM/SAH	1.1 ± 0.4	1.0 ± 0.4	1.0 ± 0.3
ADMA	0.88 ± 0.20	0.64 ± 0.17	1.1 ± 0.7
SDMA	1.1 ± 0.5	0.77 ± 0.24	0.81 ± 0.30
		HEART	
SAM	34.6 ± 3.8	30.2 ± 8.7	33.1 ± 6.5
SAH	2.3 ± 0.2	2.6 ± 0.5	21.9 ± 11.1*
SAM/SAH	15.2 ± 2.1	11.6 ± 2.5*	1.7 ± 0.7*
ADMA	2.1 ± 0.6	1.6 ± 0.3	1.8 ± 0.3
SDMA	0.44 ± 0.12	0.30 ± 0.06*	0.34 ± 0.06

nmol/g, Values expressed as mean ± SD (n=6-7)
* $p < 0.05$ Compared to CD of same region

Low folate or folate deficient diets had no effect on methylation metabolites in kidney tissue from young and old mice, with the exception of an increase in ADMA in young mice fed a folate deficient diet.

A low folate diet caused a reduced SAM/SAH ratio in heart tissues from young and old mice compared to mice fed control diet, however no significant changes in SAM or SAH were present. Heart tissues from both young and old mice reared on a folate deficient diet had SAH levels nearly 10-fold higher than mice fed a control diet, which resulted in a significant decrease in the SAM/SAH ratio.

Relative mRNA levels for SAHase, BHMT, CBS, MAT, MTHFR, MTR, MTRR, and SHMT were analyzed in liver tissues from young and old mice on the various treatment diets. The mRNA data for young mice and old mice are presented in figures 24 and 25, respectively. A folate deficient diet caused a substantial decrease in SHMT mRNA in both age groups compared to the control diet groups. Additionally, young mice reared on a folate deficient diet had elevated MAT 1A mRNA levels compared to young mice fed a control diet. In contrast MAT 1A mRNA levels were not altered in old mice fed a folate deficient diet, but MTRR mRNA levels were reduced compared to old mice fed a control diet.

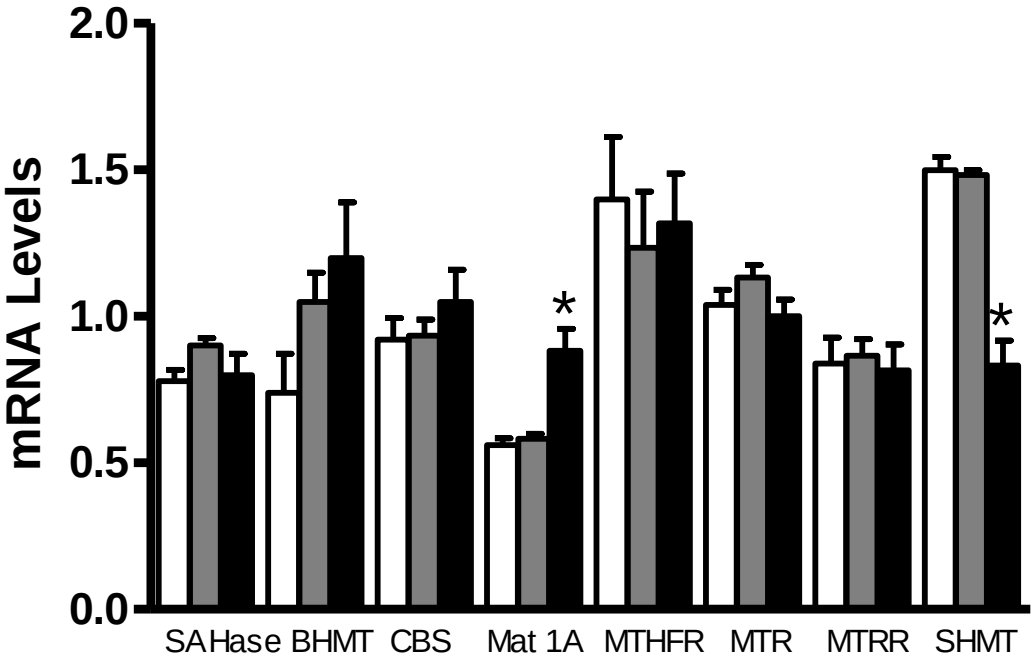


Figure 24. Liver mRNA levels of various enzymes involved in the methylation and transsulfuration pathways from young mice sacrificed by CO_2 asphyxiation: effect of folate. Data presented as mean ± SD. White bars represent CD, grey bars represent LF, and black bars represent FD mice. *$p<0.05$ (n=5).

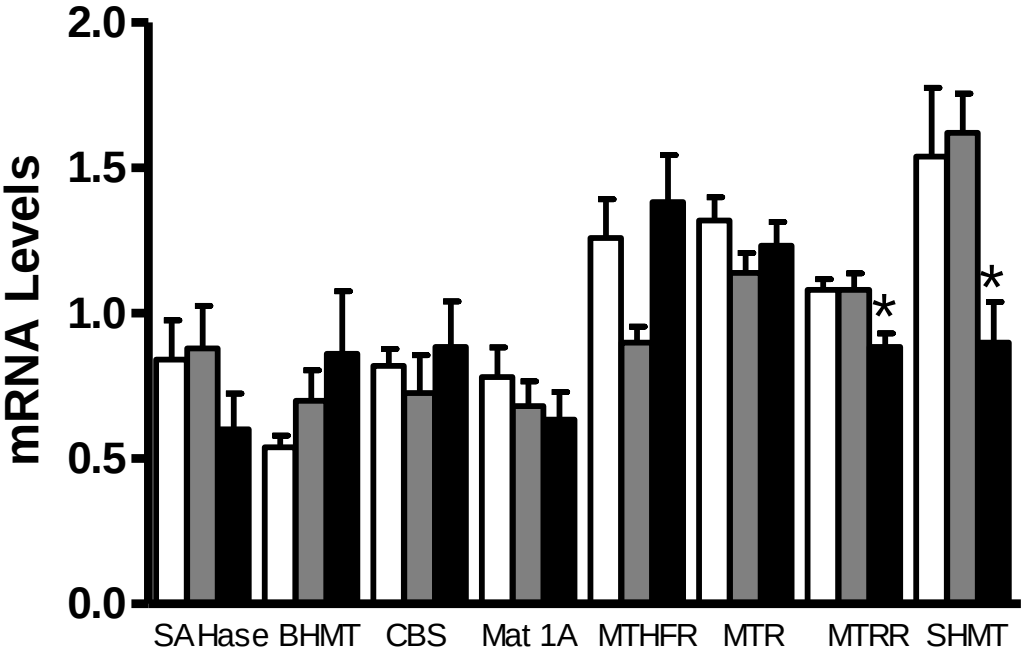


Figure 25. Liver mRNA levels of various enzymes involved in the methylation and transsulfuration pathways from old mice sacrificed by CO_2 asphyxiation: effect of folate. Data presented as mean ± SD. White bars represent CD, grey bars represent LF, and black bars represent FD mice. *$p<0.05$ (n=5).

The various brain region methylation metabolite concentrations in young mice are shown in tables 21 and 22. The only significant observation in young mice fed a low folate diet was an elevation in SDMA in the cortex compared to control diet fed mice. A folate deficient diet resulted in more drastic shifts to the methylation metabolites in young mice. Young mice fed a folate deficient diet had a significant decrease in SAM in the hippocampus, cerebellum, and mid-brain whereas SAH was increased in all regions but this was only statistically significant in the frontal cortex, cortex, and cerebellum compare to control diet mice of the same age. The SAM/SAH ratio was impaired by approximately half in all brain regions analyzed except for the striatum in young mice reared on a folate deficient diet compared to control diet mice. In addition, young mice

Table 21. Brain tissue (striatum, hippocampus, and frontal cortex) methylation metabolites from young mice sacrificed by CO_2 asphyxiation: effect of folate.

	YOUNG		
Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
	STRIATUM		
SAM	16.4 ± 1.8	14.8 ± 3.6	16.9 ± 2.4
SAH	9.3 ± 0.7	9.1 ± 3.0	10.8 ± 3.6
SAM/SAH	1.8 ± 0.3	1.7 ± 0.3	1.7 ± 0.5
ADMA	0.21 ± 0.02	0.22 ± 0.06	0.22 ± 0.05
SDMA	0.32 ± 0.07	0.32 ± 0.12	0.34 ± 0.08
	HIPPOCAMPUS		
SAM	21.8 ± 1.9	18.8 ± 2.5	17.8 ± 1.9*
SAH	3.1 ± 0.6	2.7 ± 0.5	4.2 ± 1.8
SAM/SAH	7.4 ± 1.7	7.1 ± 1.0	4.7 ± 1.4*
ADMA	0.29 ± 0.02	0.26 ± 0.04	0.29 ± 0.11
SDMA	0.35 ± 0.02	0.34 ± 0.05	0.35 ± 0.10
	FRONTAL CORTEX		
SAM	18.3 ± 2.1	19.1 ± 1.1	18.1 ± 1.6
SAH	3.8 ± 0.9	4.9 ± 1.0	7.7 ± 3.2*
SAM/SAH	5.1 ± 1.3	4.1 ± 0.9	2.7 ± 0.9*
ADMA	0.23 ± 0.05	0.27 ± 0.04	0.29 ± 0.04*
SDMA	0.28 ± 0.03	0.36 ± 0.09	0.39 ± 0.10*

nmol/g, Values expressed as mean ± SD (CD & LF n=7-8; FD n=13-16)
* $p < 0.05$ Compared to CD of same region

fed a folate deficient diet had remarkably reduced betaine levels and increased cystathionine levels in all three brain region tissues (cortex, cerebellum, and mid-brain) studied for these metabolites. Young mice that consumed a folate deficient diet had a decrease in methionine in the cerebellum and mid-brain compared to mice fed adequate folate. Lastly, ADMA and SDMA were elevated in the frontal cortex and cortex of young mice fed folate deficient diet compared to age-matched mice fed a control diet.

Poor folate status produced very similar methylation metabolite alterations in brain tissues from old mice compared to young mice. Methylation metabolites measured in CNS tissues from old mice on treatment diets is presented in tables 23 and 24. A low

Table 22. Brain tissue (cortex, cerebellum, and mid-brain) methylation metabolites from young mice sacrificed by CO_2 asphyxiation: effect of folate.

	YOUNG		
Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
		CORTEX	
SAM	16.5 ± 1.5	17.1 ± 1.2	16.2 ± 3.2
SAH	3.3 ± 0.4	3.7 ± 0.4	6.1 ± 2.4*
SAM/SAH	5.0 ± 0.4	4.7 ± 0.7	3.0 ± 1.3*
CYSTA	23.1 ± 2.6	40.9 ± 3.4	331.3 ± 198.3*
MET	62.6 ± 8.0	56.6 ± 8.3	45.8 ± 27.9
BET	12.5 ± 4.5	12.1 ± 3.4	5.8 ± 1.5*
ADMA	0.19 ± 0.01	0.19 ± 0.02	0.25 ± 0.07*
SDMA	0.26 ± 0.02	0.30 ± 0.03*	0.33 ± 0.05*
		CEREBELLUM	
SAM	21.5 ± 1.5	19.8 ± 1.2	18.2 ± 2.3*
SAH	2.9 ± 0.3	2.9 ± 0.4	4.4 ± 1.6*
SAM/SAH	7.5 ± 1.0	6.9 ± 0.9	4.8 ± 1.8*
CYSTA	92.0 ± 12.1	138.3 ± 21.8	1223 ± 505*
MET	80.6 ± 16.3	70.2 ± 9.7	54.0 ± 21.4*
BET	34.5 ± 6.8	36.3 ± 5.4	17.4 ± 1.6*
ADMA	0.20 ± 0.01	0.21 ± 0.03	0.22 ± 0.03
SDMA	0.24 ± 0.02	0.26 ± 0.02	0.28 ± 0.06
		MID-BRAIN	
SAM	18.1 ± 5.7	14.6 ± 5.0	12.8 ± 4.8*
SAH	3.6 ± 0.9	2.8 ± 0.7	4.5 ± 3.0
SAM/SAH	4.9 ± 0.7	5.1 ± 0.7	3.3 ± 1.1*
CYSTA	41.8 ± 15.7	49.0 ± 19.1	500.8 ± 481.2*
MET	52.7 ± 19.5	41.9 ± 14.5	31.8 ± 11.5*
BET	22.6 ± 9.8	18.7 ± 8.3	8.9 ± 3.4*
ADMA	0.21 ± 0.03	0.19 ± 0.04	0.19 ± 0.04
SDMA	0.36 ± 0.08	0.36 ± 0.11	0.36 ± 0.15

nmol/g, Values expressed as mean ± SD (CD & LF n=7-8; FD n=13-16)
* $p < 0.05$ Compared to CD of same region

folate diet did not cause any significant changes in the methylation metabolites analyzed. Old mice fed a folate deficient diet had reduced SAM levels in the hippocampus compared to age-matched mice fed a control diet. Interestingly, SAH levels were increased in more regions in the old deficient mice than young. Nearly a 50% decrease in the SAM/SAH ratio due to increased SAH occurred in old mice fed a folate deficient diet.

Table 23. Brain tissue (striatum, hippocampus, and frontal cortex) methylation metabolites from old mice sacrificed by CO_2 asphyxiation: effect of folate.

	OLD		
Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
	STRIATUM		
SAM	16.5 ± 2.7	16.8 ± 3.3	14.2 ± 3.1
SAH	9.5 ± 1.8	9.6 ± 2.3	9.0 ± 1.1
SAM/SAH	1.7 ± 0.2	1.9 ± 0.9	1.6 ± 0.3
ADMA	0.24 ± 0.03	0.22 ± 0.05	0.24 ± 0.07
SDMA	0.46 ± 0.13	0.45 ± 0.09	0.46 ± 0.07
	HIPPOCAMPUS		
SAM	22.0 ± 3.0	20.5 ± 1.6	16.9 ± 3.7*
SAH	3.0 ± 0.3	2.6 ± 0.2	4.9 ± 1.3*
SAM/SAH	7.3 ± 1.0	7.8 ± 0.5	3.6 ± 0.7*
ADMA	0.31 ± 0.04	0.25 ± 0.03	0.30 ± 0.07
SDMA	0.47 ± 0.10	0.42 ± 0.04	0.44 ± 0.07
	FRONTAL CORTEX		
SAM	18.0 ± 0.8	17.1 ± 3.7	16.7 ± 2.4
SAH	4.0 ± 0.7	5.3 ± 2.2	6.8 ± 1.5*
SAM/SAH	4.6 ± 1.0	3.6 ± 1.5	2.5 ± 0.7*
ADMA	0.47 ± 0.30^	0.26 ± 0.09	0.30 ± 0.09
SDMA	0.44 ± 0.05^	0.44 ± 0.05	0.40 ± 0.06

nmol/g, Values expressed as mean ± SD (n=4-7)
* $p < 0.05$ Compared to CD of same region

In old mice fed a folate deficient diet betaine was decreased in the cerebellum and mid-brain, whereas cystathionine was increased in the cortex, cerebellum, and mid-brain; very similar to the results from the young folate deficient mice. The metabolite methionine was elevated in the cerebellum and mid-brain of old folate deficient mice, the exact opposite of what occurred in young folate deficient mice. Lastly, cortical levels of ADMA and SDMA were elevated in old mice fed a folate deficient diet compared to mice on control diet. Table 25 summarizes the effects of folate deficiency on methylation metabolites analyzed in this work in the various brain regions from young and old mice.

Table 24. Brain tissue (cortex, cerebellum, and mid-brain) methylation metabolites from old mice sacrificed by CO_2 asphyxiation: effect of folate.

	OLD		
Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
		CORTEX	
SAM	16.5 ± 1.3	17.2 ± 1.5	16.0 ± 2.1
SAH	3.6 ± 0.4	4.0 ± 0.7	7.3 ± 1.4*
SAM/SAH	4.7 ± 0.7	4.3 ± 0.9	2.3 ± 0.5*
CYSTA	30.6 ± 5.0	41.6 ± 3.0	473.3 ± 208.5*
MET	63.3 ± 13.3	55.6 ± 4.2	85.6 ± 30.9
BET	14.9 ± 4.8	11.5 ± 2.8	8.8 ± 7.6
ADMA	0.22 ± 0.03	0.24 ± 0.02	0.37 ± 0.06*
SDMA	0.36 ± 0.04	0.37 ± 0.03	0.46 ± 0.07*
		CEREBELLUM	
SAM	20.3 ± 4.5	20.0 ± 1.8	18.3 ± 2.4
SAH	3.0 ± 0.89	2.7 ± 0.7	7.6 ± 1.4*
SAM/SAH	6.9 ± 1.3	7.6 ± 1.8	2.5 ± 0.5*
CYSTA	81.0 ± 18.8	115.1 ± 11.5	1474 ± 568*
MET	71.9 ± 18.4	64.0 ± 9.7	116.9 ± 26.9*
BET	36.8 ± 6.3	28.5 ± 7.1	17.8 ± 11.5*
ADMA	0.23 ± 0.08	0.21 ± 0.01	0.61 ± 0.06*
SDMA	0.34 ± 0.08	0.32 ± 0.02	0.49 ± 0.05*
		MID-BRAIN	
SAM	14.8 ± 3.6	16.3 ± 3.7	18.8 ± 3.0
SAH	3.2 ± 0.6	3.5 ± 0.5	6.5 ± 1.0*
SAM/SAH	4.6 ± 0.6	4.6 ± 0.8	3.0 ± 0.8*
CYSTA	51.1 ± 19.8	64.2 ± 18.6	618.2 ± 291.1*
MET	48.8 ± 16.8	47.0 ± 10.0	86.3 ± 26.6*
BET	23.8 ± 8.8	19.7 ± 4.9	14.1 ± 6.7*
ADMA	0.21 ± 0.03	0.21 ± 0.03	0.30 ± 0.04*
SDMA	0.46 ± 0.13	0.48 ± 0.08	0.59 ± 0.09

nmol/g, Values expressed as mean ± SD (n=6-7)
* $p < 0.05$ Compared to CD of same region

Effect of folate deficiency in young mice sacrificed by microwave radiation. As previously mentioned the microwave radiation technique worked properly in young mice and did not work in old mice. Therefore, only microwave data from the young mice will be discussed in relation to the effect of folate deficiency on methylation metabolites. Data from young mice on the three treatment diets is shown in tables 26 and 27.

Table 25. Brain methylation metabolites summary from young and old mice sacrificed by CO_2 asphyxiation: effect of folate.

	The Effect of Folate Deficiency	
Metabolite	Young	Old
SAM	HIP,CB,MB ↓	HIP ↓
SAH	FCX,CX,CB ↑	HIP,FCX,CX,CB,MB ↑
SAM/SAH	HIP,FCX,CX,CB,MB ↓	HIP,FCX,CX,CB,MB ↓
CYSTA	CX,CB,MB ↑	CX,CB,MB ↑
MET	CB,MB ↓	CB,MB↑
BET	CX,CB,MB ↓	CB,MB ↓
ADMA	FCX,CX ↑	CX ↑
SDMA	FCX,CX ↑	CX ↑

SAM concentrations were decreased in all six brain regions from mice fed a folate deficient diet compared to mice fed a control diet, but only to a statistically significant degree in the frontal cortex and cerebellum. Mice fed a folate deficient diet sacrificed by microwave radiation did not show the same increase in SAH levels as the folate deficient mice sacrificed by CO_2 asphyxiation. Only the mid-brain had increased SAH levels in mice fed a folate deficient diet compared to control diet mice. The SAM/SAH ratio was decreased in four regions (striatum, frontal cortex, cerebellum, and mid-brain) due to folate deficiency. Under a folate deprived state the metabolites, cystathionine, methionine, and betaine were altered in all brain regions studied, thus these metabolites are extremely sensitive to mode of sacrifice. Cystathionine was increased and betaine and methionine decreased in mice fed a folate deficient diet. Young folate deficient mice sacrificed by microwave radiation had reduced choline levels in the mid-brain compared to control diet mice sacrificed by the same method. Unlike folate deficient mice sacrificed by CO_2 asphyxiation, folate deficient mice sacrificed by microwave radiation showed no changes in ADMA and SDMA in brain tissues. Table 28 summarizes the

effects of folate deficiency on methylation metabolites in young mice sacrificed by microwave radiation.

Table 26. Brian tissue (striatum, hippocampus, and frontal cortex) methylation metabolites from young mice sacrificed by microwave radiation: effect of folate.

	YOUNG		
Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
	STRIATUM		
SAM	12.2 ± 2.3	10.6 ± 3.9	8.9 ± 3.2
SAH	0.33 ± 0.15	0.30 ± 0.08	0.46 ± 0.14
SAM/SAH	44.5 ± 22.8	37.1 ± 14.8	19.6 ± 5.4*
ADE	1.5 ± 0.9	1.0 ± 0.3	1.9 ± 0.8
CYSTA	48.8 ± 7.3	77.0 ± 7.2	834.2 ± 358*
MET	80.2 ± 9.3	76.3 ± 8.1	51.8 ± 12.9*
BET	41.1 ± 7.5	27.4 ± 14.1	12.8 ± 5.7*
CHO	36.8 ± 8.6	35.3 ± 3.5	33.4 ± 7.6
ADMA	0.22 ± 0.02	0.19 ± 0.03	0.21 ± 0.03
SDMA	0.42 ± 0.05	0.38 ± 0.07	0.37 ± 0.04
	HIPPOCAMPUS		
SAM	8.4 ± 2.7	8.5 ± 3.4	4.98 ± 2.92
SAH	0.31 ± 0.11	0.31 ± 0.07	0.38 ± 0.12
SAM/SAH	31.7 ± 16.4	29.4 ± 14.8	13.5 ± 6.3
ADE	1.1 ± 0.4	0.99 ± 0.58	1.7 ± 0.5
CYSTA	46.3 ± 9.2	55.1 ± 12.1	709.5 ± 361.0*
MET	63.0 ± 7.0	56.8 ± 11.4	40.1 ± 12.1*
BET	39.3 ± 8.7	25.0 ± 7.3*	18.4 ± 4.1*
CHO	26.4 ± 7.3	23.9 ± 7.2	23.4 ± 3.3
ADMA	0.36 ± 0.04	0.30 ± 0.02	0.37 ± 0.07
SDMA	0.24 ± 0.06	0.19 ± 0.06	0.24 ± 0.03
	FRONTAL CORTEX		
SAM	15.1 ± 1.0	14.8 ± 3.4	11.5 ± 1.7*
SAH	0.21 ± 0.07	0.35 ± 0.25	0.35 ± 0.10
SAM/SAH	78.3 ± 24.5	62.3 ± 47.3	35.5 ± 10.7*
ADE	1.3 ± 0.5	1.5 ± 0.6	1.5 ± 0.7
CYSTA	39.3 ± 4.9	45.5 ± 3.0	670.7 ± 389.6*
MET	64.7 ± 5.2	61.9 ± 6.4	43.8 ± 11.4*
BET	32.7 ± 7.1	13.3 ± 3.2*	10.4 ± 2.8*
CHO	28.1 ± 2.2	20.9 ± 8.2	20.1 ± 3.3
ADMA	0.24 ± 0.04	0.27 ± 0.06	0.23 ± 0.06
SDMA	0.34 ± 0.05	0.25 ± 0.04*	0.29 ± 0.07

nmol/g, Values expressed as mean ± SD (n=5-6)

* $p < 0.05$ Compared to CD of same region

Table 27. Brain tissue (cortex, cerebellum, mid-brain) methylation metabolites from young mice sacrificed by microwave radiation: effect of folate.

	YOUNG		
Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
		CORTEX	
SAM	14.3 ± 4.6	13.7 ± 3.4	11.3 ± 2.7
SAH	0.28 ± 0.17	0.30 ± 0.16	0.34 ± 0.19
SAM/SAH	65.2 ± 33.2	67.1 ± 61.4	46.8 ± 31.2
ADE	1.6 ± 0.6	2.3± 1.0	1.8 ± 0.4
CYSTA	36.1 ± 7.4	58.7 ± 6.2	734.70 ± 368.3*
MET	70.2 ± 11.6	70.9 ± 13.5	47.3 ± 16.4*
BET	30.1 ± 6.8	20.1 ± 7.2	10.4 ± 6.4*
CHO	30.8 ± 6.7	30.7 ± 7.2	25.1 ± 4.6
ADMA	0.38 ± 0.03	0.38 ± 0.05	0.40 ± 0.06
SDMA	0.38 ± 0.07	0.35 ± 0.02	0.38 ± 0.02
		CEREBELLUM	
SAM	18.1 ± 3.2	20.5 ± 3.8	11.7 ± 2.2*
SAH	0.33 ± 0.04	0.39 ± 0.04	0.52 ± 0.19
SAM/SAH	54.9 ± 9.5	53.2 ± 11.9	24.5 ± 7.9*
ADE	2.0 ± 1.1	2.3 ± 1.3	2.3 ± 1.5
CYSTA	96.8 ± 11.1	163.6 ± 13.3	1946 ± 697*
MET	69.7 ± 6.0	73.8 ± 13.3	47.8 ± 12.3*
BET	57.8 ± 5.5	37.6 ± 14.6*	16.2 ± 4.8*
CHO	34.7 ± 12.2	40.6 ± 10.9	22.2 ± 2.8
ADMA	0.27 ± 0.03	0.27 ± 0.01	0.27 ± 0.03
SDMA	0.30 ± 0.06	0.28 ± 0.03	0.31 ± 0.06
		MID-BRAIN	
SAM	12.4 ± 3.5	12.4 ± 3.0	7.61 ± 3.97
SAH	0.28 ± 0.03	0.32 ± 0.02	0.42 ± 0.09*
SAM/SAH	44.1 ± 12.7	39.4 ± 11.3	18.1 ± 9.4*
ADE	1.3 ± 0.6	1.9 ± 0.7	1.2 ± 0.5
CYSTA	57.5 ± 7.4	86.4 ± 7.8	1074 ± 445*
MET	44.1 ± 12.7	59.6 ± 8.6	38.3 ± 16.6*
BET	42.0 ± 10.0	29.8 ± 15.3	9.1 ± 5.0*
CHO	33.6 ± 5.8	32.9 ± 3.7	22.3 ± 4.6*
ADMA	0.14 ± 0.01	0.13 ± 0.02	0.15 ± 0.03
SDMA	0.35 ± 0.06	0.33 ± 0.04	0.34 ± 0.06

nmol/g, Values expressed as mean ± SD (n=4-6)

* $p < 0.05$ Compared to CD of same region

Table 28. Brain tissue methylation metabolite summary from young mice sacrificed by microwave radiation: effect of folate.

	The Effect of Folate Deficiency
Metabolite	Young
SAM	FCX,CB ↓
SAH	MB ↑
SAM/SAH	STR,FCX,CB,MB ↓
ADE	none
CYSTA	STR,HIP,FCX,CX,CB,MB ↑
MET	STR,HIP,FCX,CX,CB,MB ↓
BET	STR,HIP,FCX,CX,CB,MB ↓
CHO	MB ↓
ADMA	none
SDMA	none

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Effect of low folate on weight. Female *tg*-MTHFR mice weighed significantly less than males at baseline. During treatment females gained weight while the males decreased in weight, thereby there was slightly less of a difference after 6 months of treatment than at start of treatment (Figure 26). Genotype, low folate, and a combination of the two did not influence weight in male or females (data not shown).

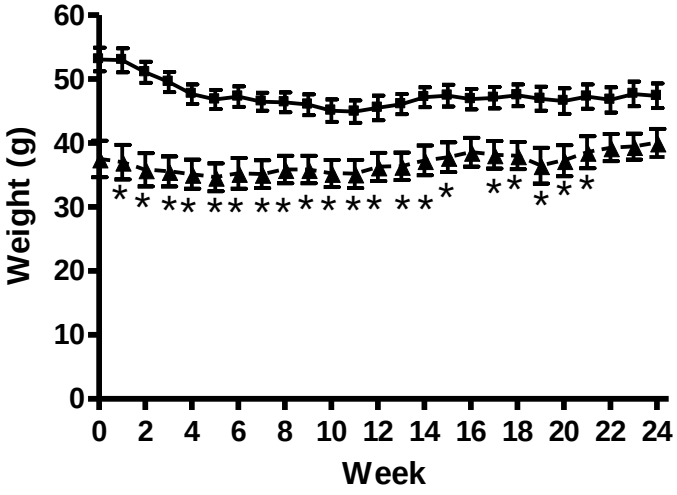

Figure 26. Effect of gender on growth in *tg*-MTHFR mice. Data presented as mean ± SEM ■=Male (n=10), ▲=Female (n=10).

Effect of gender. Gender did not affect plasma 5-MTHF or tHcy levels in *tg*-MTHFR mice. A summary of the methylation metabolite differences between male and female *tg*-MTHFR mice is shown in table 29. Methionine was the only methylation metabolite that had gross differences in more than one brain region, with females having increased levels compared to males in the striatum, frontal cortex, cortex, and mid-brain. Additionally, cortical levels of SAM, SAH and the SAM/SAH ratio were altered between males and females. Due to the fact that there was no variance in plasma 5-MTHF and tHcy and only minor differences in methylation metabolite concentrations between male and female *tg*-MTHFR mice, only the male data will be assessed in the remainder of this chapter.

Table 29. Brain tissue methylation metabolite summary: effect of gender.

Metabolite	The Effect of Gender
5-MTHF (nmol/L)	none
Hcy	none
SAM	Female: CX ↓
SAH	Female: CX ↑
SAM/SAH	Female: CX ↓
CYSTA	none
MET	Female: STR,FCX,CX,MB ↑
BET	none
ADMA	none
SDMA	none

Effect of genotype and low folate in mice sacrificed by CO_2 asphyxiation. The methylation metabolites that were analyzed from plasma of *tg*-MTHFR mice are presented in table 30. Heterozygous mice had a decrease in 5-MTHF levels compared to wild type littermates. Similarly, tHcy levels were also affected by genotype; heterozygous mice had increased concentrations compared to wild type mice.

Table 30. Plasma methylation metabolites from *tg*-MTHFR mice: effect of genotype and folate.

Metabolite	Control Diet WT	Low Folate Diet WT	Control Diet HET	Low Folate Diet HET
5-MTHF (nmol/L)	85.5 ± 40.9	6.70 ± 3.18*	39.9 ± 12.0*	5.61 ± 3.15*#
tHCY	5.1 ± 0.5	37.4 ± 17.9*	11.4 ± 2.6*	59.7 ± 13.9*#^
SAM	0.31 ± 0.09	0.20 ± 0.05*	0.36 ± 0.13	0.19 ± 0.02*#
SAH	0.09 ± 0.05	0.20 ± 0.10*	0.24 ± 0.20	0.34 ± 0.20*#
SAM/SAH	3.7 ± 1.1	1.8 ± 2.5	2.1 ± 1.3	0.69 ± 0.25*#
CYSTA	1.1 ± 0.43	2.7 ± 1.6*	1.3 ± 0.3	4.3 ± 1.0*#^
MET	100.7 ± 31.1	58.3 ± 15.6*	86.4 ± 12.1	52.4 ± 16.4*#
BET	41.4 ± 8.6	21.0 ± 6.4*	45.1 ± 23.1	16.5 ± 3.6*#
ADMA	0.54 ± 0.11	0.47 ± 0.13	0.54 ± 0.16	0.48 ± 0.07
SDMA	0.14 ± 0.03	0.12 ± 0.03	0.16 ± 0.06	0.13 ± 0.02

µmol/L, Values expressed as mean ± SD (n=5-10)

* $p < 0.05$ Compared to CD WT

# $p < 0.05$ Compared to CD HET

^ $p < 0.05$ Compared to LF WT

Mice fed a low folate diet had a decrease in 5-MTHF and an increase in tHcy concentrations compared to mice fed a control diet. The change in tHcy was exacerbated in the heterozygous genotype reared on a low folate diet compared to wild type reared on the same diet. Low folate status did decrease SAM and increase SAH; however, the SAM/SAH ratio was not affected due to these alterations in wild type mice. Plasma cystathionine levels were elevated in mice fed a low folate diet, while methionine and betaine levels were decreased. The combination of a heterozygous genotype and low folate diet did not exacerbate the effects observed in SAM, SAH, methionine, or betaine but caused a substantial difference in cystathionine. Cystathionine levels were doubled in heterozygous mice fed a low folate diet compared to wild type mice fed the same diet. Lastly, heterozygous mice fed a low folate diet were the only group that had a decrease in

the SAM/SAH ratio. The methylation metabolites choline, ADMA, and SDMA were not affected by rearing on a low folate diet.

The methylation metabolites investigated in the liver of *tg*-MTHFR mice are presented in table 31. Unlike in plasma there were a few differences between the two genotypes in SAM and betaine levels in the liver. The heterozygous mice showed significant decreases in SAM, SAM/SAH, and betaine in liver tissue compared to wild type mice. The treatment diet (low folate) also invoked similar changes in these same metabolites. The combination of heterozygosity and a low folate diet did not result in statistically significant changes from wild type mice fed a low folate diet. The only additional difference in this group was that they also presented with a decrease in methionine.

Table 31. Liver methylation metabolites from *tg*-MTHFR mice: effect of genotype and folate.

Metabolite	Control Diet WT	Low Folate Diet WT	Control Diet HET	Low Folate Diet HET
SAM	56.1 ± 19.2	20.3 ± 8.8*	37.9 ± 7.3*	15.8 ± 6.5*#
SAH	15.1 ± 6.8	38.9 ± 22.2*	16.2 ± 2.7	36.1 ± 17.1*#
SAM/SAH	4.2 ± 1.6	0.62 ± 0.38*	2.4 ± 0.5*	0.46 ± 0.12*#
CYSTA	33.2 ± 18.1	29.1 ± 19.4	29.5 ± 10.6	27.8 ± 6.3
MET	21.1 ± 8.1	14.8 ± 5.0	20.8 ± 10.4	11.9 ± 5.8*#
BET	261.3 ± 62.8	46.7 ± 22.0*	139.7 ± 78.3*	36.1 ± 18.0*#
ADMA	0.70 ± 0.12	0.56 ± 0.27	0.48 ± 0.34	0.62 ± 0.17
SDMA	0.46 ± 0.16	0.38 ± 0.16	0.37 ± 0.10	0.39 ± 0.12

nmol/L, Values expressed as mean ± SD (n=6-10)
* $p < 0.05$ Compared to CD WT
# $p < 0.05$ Compared to CD HET
^ $p < 0.05$ Compared to LF WT

Tables 32 and 33 reports the levels of the methylation metabolites in the six brain regions studied from the *tg*-MTHFR aged mouse model in this dissertation. Methionine

was slightly down in heterozygous mice compared to the wild type controls, however, only to a significant degree in the hippocampus. Cystathionine levels were elevated nearly 2-fold in four of the six brain regions in heterozygous mice compared to wild type mice.

Table 32. Brain tissue (striatum, hippocampus, and frontal cortex) methylation metabolites from *tg*-MTHFR mice: effect of genotype and folate.

Metabolite	Control Diet WT	Low Folate Diet WT	Control Diet HET	Low Folate Diet HET
		STRIATUM		
SAM	18.2 ± 3.3	17.8 ± 3.9	19.9 ± 4.0	17.2 ± 2.5
SAH	5.9 ± 1.5	6.6 ± 1.2	5.9 ± 2.0	7.3 ± 1.2*
SAM/SAH	3.2 ± 0.9	2.8 ± 0.6	4.1 ± 2.9	2.4 ± 0.5*
CYSTA	55.0 ± 15.1	141.0 ± 31.2*	67.9 ± 17.4	258.7 ± 125.9 ^*#
MET	91.0 ± 21.1	84.4 ± 22.2	82.7 ± 18.1	62.6 ± 7.6 #
BET	18.9 ± 7.7	14.6 ± 8.1	18.0 ± 8.9	13.5 ± 4.2
ADMA	0.22 ± 0.04	0.23 ± 0.09	0.23 ± 0.07	0.21 ± 0.04
SDMA	0.33 ± 0.05	0.36 ± 0.05	0.35 ± 0.08	0.32 ± 0.03
		HIPPOCAMPUS		
SAM	21.3 ± 3.0	19.9 ± 3.8	18.1 ± 2.3	18.6 ± 4.1
SAH	1.4 ± 0.4	1.4 ± 0.3	1.5 ± 0.6	1.6 ± 0.4
SAM/SAH	16.6 ± 4.5	14.2 ± 2.3	13.0 ± 4.3	12.1 ± 3.0*
CYSTA	44.7 ± 5.5	123.7 ± 49.0*	52.7 ± 11.4	255.3 ± 88.3^*#
MET	80.0 ± 14.8	66.8 ± 12.5	56.6 ± 3.1*	50.6 ± 9.3^*
BET	10.1 ± 2.7	5.6 ± 4.1*	5.4 ± 6.6	3.5 ± 2.6*
ADMA	0.26 ± 0.05	0.26 ± 0.08	0.24 ± 0.06	0.24 ± 0.06
SDMA	0.36 ± 0.04	0.37 ± 0.08	0.33 ± 0.05	0.33 ± 0.08
		FRONTAL CORTEX		
SAM	19.8 ± 3.2	21.0 ± 2.1	21.5 ± 3.9	21.1 ± 3.0
SAH	1.16 ± 0.46	1.55 ± 0.44	1.43 ± 0.29	2.41 ± 0.51^*#
SAM/SAH	18.7 ± 5.9	15.0 ± 6.5	15.7 ± 4.9	9.04 ± 1.92^*#
CYSTA	22.8 ± 4.6	64.4 ± 26.2*	40.1 ± 6.0*	192.0 ± 73.6^*#
MET	75.6 ± 14.6	75.9 ± 16.5	70.6 ± 8.8	61.2 ± 12.7*
BET	13.0 ± 4.2	11.0 ± 5.8	13.9 ± 3.4	10.2 ± 4.3
ADMA	0.24 ± 0.06	0.29 ± 0.04	0.29 ± 0.06	0.28 ± 0.06
SDMA	0.31 ± 0.05	0.36 ± 0.05	0.32 ± 0.05	0.38 ± 0.06

nmol/L, Values expressed as mean ± SD (n=3-9)
* $p < 0.05$ Compared to CD WT
# $p < 0.05$ Compared to CD HET
^ $p < 0.05$ Compared to LF WT

Table 33. Brain tissue (cortex, cerebellum, and mid-brain) methylation metabolites from *tg*-MTHFR mice: effect of genotype and folate.

Metabolite	Control Diet WT	Low Folate Diet WT	Control Diet HET	Low Folate Diet HET
		CORTEX		
SAM	20.2 ± 3.0	13.4 ± 4.1*	18.9 ± 4.5	16.4 ± 3.9*
SAH	2.8 ± 0.5	4.2 ± 1.1*	2.9 ± 0.7	4.7 ± 0.7*#
SAM/SAH	7.5 ± 1.4	3.7 ± 2.2*	7.1 ± 2.6	3.7 ± 1.3*#
CYSTA	27.2 ± 7.4	74.3 ± 24.1*	42.4 ± 5.0*	201.1 ± 85.3^*#
MET	85.2 ± 19.4	103.0 ± 34.6	78.3 ± 20.1	79.5 ± 36.0
BET	14.8 ± 3.7	10.0 ± 3.5*	13.5 ± 2.7	10.5 ± 1.9*#
ADMA	0.19 ± 0.06	0.17 ± 0.05	0.16 ± 0.04	0.15 ± 0.04
SDMA	0.29 ± 0.04	0.27 ± 0.03	0.30 ± 0.05	0.28 ± 0.03
		CEREBELLUM		
SAM	23.8 ± 3.3	25.2 ± 2.4	24.2 ± 4.5	23.3 ± 2.34
SAH	3.0 ± 0.9	3.4 ± 0.6	3.0 ± 0.8	4.13 ± 0.37^*#
SAM/SAH	8.4 ± 2.6	7.6 ± 1.0	8.2 ± 1.5	5.7 ± 0.3^*#
CYSTA	71.8 ± 12.1	316.2 ± 150.1*	117.9 ± 31.0*	517.3 ± 210.4*#
MET	87.3 ± 27.7	82.7 ± 12.6	66.5 ± 19.7	68.9 ± 8.8^
BET	42.3 ± 9.7	35.7 ± 9.2	37.3 ± 12.4	32.3 ± 4.7*
ADMA	0.33 ± 0.14	0.30 ± 0.04	0.28 ± 0.09	0.29 ± 0.04
SDMA	0.34 ± 0.04	0.37 ± 0.04	0.34 ± 0.10	0.35 ± 0.01
		MID-BRAIN		
SAM	22.1 ± 1.3	20.8 ± 2.4	21.4 ± 2.2	21.1 ± 1.7
SAH	1.7 ± 0.4	1.8 ± 0.3	2.0 ± 0.5	2.6± 0.7^*
SAM/SAH	13.5 ± 3.4	11.9 ± 2.0	11.4 ± 2.4	8.6 ± 2.0^*#
CYSTA	49.0 ± 6.2	117.7 ± 32.4*	69.0 ± 20.6*	234.3 ± 58.7^*#
MET	62.9 ± 10.5	50.0 ± 8.7*	53.0 ± 9.5	48.0 ± 9.3*
BET	28.2 ± 7.2	20.7 ± 6.1	25.8 ± 12.9	23.9 ± 7.5
ADMA	0.22 ± 0.04	0.17 ± 0.03*	0.21 ± 0.07	0.19 ± 0.03
SDMA	0.38 ± 0.06	0.38 ± 0.08	0.38 ± 0.08	0.41 ± 0.04

nmol/L, Values expressed as mean ± SD (n=6-10)
* $p < 0.05$ Compared to CD WT
# $p < 0.05$ Compared to CD HET
^ $p < 0.05$ Compared to LF WT

In cortex brain tissues SAM levels were reduced and SAH levels increased in wild type mice fed a low folate diet for 6 months compared to those reared on a control diet. Heterozygosity in addition to a low folate diet did not exacerbate the decrease in SAM levels or the increase in SAH levels in this brain region. Furthermore, the cortex

was the only brain region to have significant changes in SAM, SAH, and the SAM/SAH ratio due to folate status alone. On the other hand SAH was drastically affected by the combination of a heterozygous genotype and a low folate diet in other brain regions. Increased SAH was present in the striatum, frontal cortex, cortex, cerebellum, and mid-brain of *tg*-MTHFR heterozygous mice reared on a low folate diet compared to wild type mice fed a control diet. Similarly, the SAM/SAH ratio was significantly decreased in all six brain regions of *tg*-MTHFR heterozygous mice fed a low folate diet compared to *tg*-MTHFR wild type mice fed a control diet. Also noteworthy is that SAM levels do not vary much by region but SAH levels do vary, with the highest levels being found in the striatum and therefore so is the SAM/SAH ratio.

Cystathionine was significantly elevated in *tg*-MTHFR wild type mice fed a low folate diet in all brain regions assessed. This change was intensified in *tg*-MTHFR heterozygous mice fed a low folate diet. Mid-brain methionine levels were depleted in wild type mice fed a low folate diet; no other regions were affected in this group. Likewise, heterozygous mice fed low folate diet showed similar decreased methionine levels in the mid-brain; in addition they also had remarkably lower levels in the hippocampus and frontal cortex. Betaine levels were decreased in the hippocampus and cortex of both wild type and heterozygous mice fed a low folate diet compared to wild type mice fed a control diet. Heterozygous mice fed a low folate diet also showed a decrease in the levels of betaine in the cerebellum. Lastly, ADMA was reduced in the mid-brain of wild type mice reared on a low folate diet but not in heterozygous mice fed the same diet. Table 34 summarizes all the changes in the methylation metabolites in *tg*-MTHFR mice.

Table 34. Brain tissue methylation metabolite summary from *tg*-MTHFR mice: effect of genotype and low folate.

	The Effect of:		
Metabolite	Genotype	Low Folate	Genotype + Low Folate
SAM	none	CX ↓	CX ↓
SAH	none	CX ↑	STR,FCX,CX,CB,MB ↑
SAM/SAH	none	CX ↓	STR,HIP,FCX,CX,CB,MB ↓
CYSTA	FCX,CX,CB,MB ↑	STR,HIP,FCX,CX,CB,MB ↑	STR,HIP,FCX,CX,CB,MB ↑
MET	HIP ↓	MB ↓	HIP,FCX,MB ↓
BET	none	HIP,CX ↓	HIP,CX,CB ↓
ADMA	none	MB ↓	none
SDMA	none	none	none

Discussion

C57BL/6J Aged Folate Deficient Study

Effect of microwave radiation. Microwave radiation caused on average a 28% decrease in SAM and a 92% decrease in SAH in brain tissues compared to mice sacrificed via CO_2 asphyxiation (Tables 15 and 16). There was also a marked increase in the SAM/SAH ratio due to microwave radiation (Tables 15 and 16). Alterations in these metabolites are likely to be due to rapid heat inactivation of methyltransferase enzymes. Microwave radiation resulted in a reduction of choline with an increase in betaine, probably due to inactivation of PEMT and BHMT, respectively (Tables 15 and 16). Choline levels might be elevated in CO_2 asphyxiated mice due to the fact that acetylcholine is rapidly exposed to post-mortem metabolism by cholinesterase, thus increasing choline levels (Bertrand et al. 1994). Regional brain (striatum, hippocampus, frontal cortex, cortex, cerebellum, and mid-brain) tissue levels of choline in mice sacrificed by both methods reported in this study are in accordance with levels reported in the literature (Ikarashi et al. 1984; Blank et al. 1979; Weintraub et al. 1976). Microwave

radiation increased ADMA and SDMA in at least three of the six brain regions assessed (Tables 15 and 16). However, ADMA was decreased in the mid-brain and SDMA in the hippocampus.

Metabolites in peripheral tissues were not evaluated for the effect of microwave radiation due to the design of the microwave instrument. The microwave beam is focused to the front of the head of the mouse, thus peripheral tissues such as liver, kidney, and heart do not receive direct radiation and heat inactivation of the enzymes in these tissues is not uniform. There are only a few publications that analyze the effects of focused microwave radiation as a method of sacrifice in rodents and the majority that do tend to focus on protein phosphorylation. This is the first investigation that documents the differences in numerous methylation metabolites in brain tissue of CO_2 asphyxiated mice versus mice sacrificed by microwave radiation.

Effect of folate deficiency. Low and folate deplete diets in young and old mice sacrificed by CO_2 asphyxiation caused mild and severe HHcy after 3 months of treatment, respectively (Tables 17 and 18). The values of plasma 5-MTHF and tHcy in rats on a control diet, low folate, and folate deficient diets for 4 weeks reported by Kim et al are nearly identical to the values reported in this dissertation (Kim et al. 1994). Another folate deficient study also reported increased plasma tHcy in mice. They also observed no changes in vitamin B_{12} or B_6 due to folate deficiency after 10 weeks of treatment (Troen et al. 2008).

Young and old mice reared on a folate deficient diet for 3 months that were sacrificed by CO_2 asphyxiation had a significantly reduced SAM/SAH ratio in plasma, liver, heart, and all brain tissue regions except for in the striatum (Tables 19-24). Young

mice sacrificed by microwave radiation also had a decrease in the SAM/SAH ratio in CNS tissues (Tables 26 and 27). A combination of a decrease in SAM and an increase in SAH was responsible for the decreased SAM/SAH ratio in many cases, however a few regions and tissues only had one of the metabolites altered thus driving the ratio independently. Interestingly, in CO_2 asphyxiated mice increased SAH was primarily responsible for the decreased SAM/SAH ratio, whereas in mice sacrificed by microwave radiation decreased SAM was primarily responsible.

The observation that HHcy induced by an inadequate supply of folate in the diet resulted in altered SAM and SAH levels and consequently a reduced SAM/SAH ratio in various tissues is consistent with previous studies (Miller et al. 1994; Sontag et al. 2008; Kim et al. 1994; Gospe et al. 1995; Pogribny et al. 2006; Fuso et al. 2008; Troen et al. 2008; Caudill et al. 2001). Manipulation of folate levels that leads to the accumulation of Hcy induces reversal of the SAHase reaction (Yi et al. 2000), and an increase in SAH. Reduced concentrations of SAM that accompany folate deficiency are due to lack of methyl groups provided by folate for the synthesis of methionine. It is noteworthy to point out that this is the first investigation of the effect of folate deficiency on methylation metabolites in an aged mouse model. Interestingly, this study determined that folate deficiency did not have a greater impact on methylation metabolites in an old mouse model. It is also the first study to investigate folate deficient induced changes in methylation metabolites in mice sacrificed by microwave radiation. Although folate deficiency induced a decreased SAM/SAH ratio in many brain region tissues in young mice sacrificed by microwave radiation similar to young mice sacrificed by CO_2 asphyxiation, the absolute concentrations of SAM and SAH were extremely different

between the two method used to sacrifice mice (Tables 21-22 and 26-27). On average brain tissue SAM levels were 2-fold and SAH levels 10-fold higher in folate deficient CO_2 asphyxiated mice. Also folate deficiency resulted in an increase in SAH in one brain region in mice sacrificed by microwave radiation, compared to three brain regions in CO_2 asphyxiated mice. This data suggests that SAH may not be as severely influenced by folate deficiency as currently proposed, but rather the increase found in studies published to date is primarily due to post-mortem events.

The reason(s) for decreased SAM/SAH ratio, whether it be increased SAH, decreased SAM, or both is important to consider because the SAM/SAH ratio is an important indicator of cellular methylation potential, it is unclear which metabolite is responsible for hypomethylation (Chiang et al. 1996; Miller et al. 1994; Sontag et al. 2008; Kim et al. 1994; Troen et al. 2008). SAH is a competitive inhibitor of SAM-dependent methyltransferase reactions and its affinity to most methyltransferase is greater than the affinity to the substrate SAM (Finkelstein 1990). Therefore an increase in SAH is presumed to be mainly responsible for decreased methylation. One study using transgenic CBS deficient mice reported SAH liver and brain tissue concentrations correlated with DNA hypomethylation when fed a methyl deficient diet for 24 weeks (Caudill et al. 2001). They also concluded that a decrease in SAM alone was not sufficient to affect DNA methylation in this model. In addition, a collaborative study between Dr. Sontag and Dr. Bottiglieri showed that a decreased SAM/SAH ratio due to increased SAH in brain tissues of mice fed a folate deplete diet for 2 months was associated with hypomethylation of the catalytic subunit of PP2A (Sontag et al. 2008). However, 4 week old rats fed a diet low in methionine and lacking folate and choline for

9 to 36 weeks presented with a decreased SAM/SAH ratio in liver tissue due to decreased SAM and was associated with global DNA hypomethylation (Pogribny et al. 2006). It is important to note that all of these studies associated a decreased SAM/SAH ratio with decreased DNA methylation. More emphasis may need to the placed on the concentrations of SAM and SAH independently rather than the SAM/SAH ratio when investigating methylation; it is possible that increased SAH may be a better indicator of methylation status than the SAM/SAH ratio in mice sacrificed by CO_2 asphyxiation. However, this may not be representative of the true physiological levels of methylation metabolites. Future studies investigating methylation in folate deficient mice sacrificed by microwave radiation would be beneficial for determining which metabolite influences methylation greater, since SAH levels are not principally elevated in many regions of brain tissue from folate deficient mice sacrificed by microwave radiation.

Cystathionine is significantly elevated in all brain regions analyzed from young mice fed a folate deficient diet sacrificed by microwave radiation (Tables 26 and 27), and young and old mice fed a folate deficient diet sacrificed by CO_2 asphyxiation (Tables 21-24). This observation is consistent with reports that up to 95% of elderly folate deficient patients have serum cystathionine levels at least 2 SD above the mean of normal blood donors (Stabler et al. 1993). Remethylation of Hcy to methionine usually predominates over the catabolic degradation of Hcy by the transsulfuration pathway (Finkelstein 2000). However, excess Hcy may be funneled into the alternate pathway in order to maintain a normal flux, thereby increasing cystathionine levels. In addition, CSL the enzyme responsible for catabolizing cystathionine is not present in brain tissue (Ishill et al. 2004).

It is unknown whether buildup of cystathionine in the brain is toxic and therefore could be of involved in the pathogenesis of diseases associated with low folate status.

Methionine levels were shown to be decreased in brain tissues from young mice reared on a folate deficient (both CO_2 asphyxiation and microwave radiated) (Tables 21-22 and 26-267). In old mice fed a folate deficient diet sacrificed by CO_2 asphyxiation brain tissue methionine levels were elevated (Tables 23 and 24). In humans it is known that the rate of synthesis of methionine is down in patients with folate deficiency due to lack of remethylation of Hcy to methionine (Cuskelly et al. 2001), this could explain the results discovered in the young folate depleted mice. To date the literature does not include any articles citing differences between methionine concentrations in young and old folate deplete mice to help draw a conclusion to the metabolism differences of methionine with regard to age. No differences in methionine were found between young and old mice fed control diet thus the changes seen between the folate deficient mice are not solely due to age. It is possible that there is an age-related increase in the enzyme activity of MTR or a decrease in MAT, only during periods of folate deficiency.

Brain and liver tissue betaine concentrations were diminished in young and old mice fed a folate deficient diet sacrificed by CO_2 asphyxiation (Tables 19-24). Additionally, levels of betaine were reduced in brain tissues from young mice fed a folate deficient diet sacrificed by microwave radiation (Tables 26 and 27). Folate deficiency did not alter betaine levels in plasma, which is consistent with other studies in humans and mice (Allen et al. 1993). They reported plasma betaine levels are maintained in the majority of patients with folate deficiency and parallel results were found in mice. The data presented in this study shows the importance of measuring tissue levels. In liver

tissues, BHMT mRNA levels were unaltered by inadequate folate (Figures 24 and 25). This finding suggests that the enzymatic activity of BHMT may not be affected or responsible for the decrease in betaine levels. In liver tissues the decrease in betaine is likely due to increased utilization as a methyl donor to Hcy. Since BHMT is absent in brain tissues this pathways cannot account for the decreased concentrations of betaine in various CNS regions. Neither can this be explained by reduced uptake from the circulation since plasma levels are not affected. Moreover, one source of choline is derived from breakdown of phosphatidylcholine a product of methyltransferase reactions. If hypomethylation occurred in this model as suggested by reduced SAM/SAH ratio, phosphatidylcholine levels could possibly be decreased resulting in decreased choline and betaine levels. A 10 week long folate deficient study in rats revealed decreased phosphatidylcholine levels in brain membranes (Troen et al. 2008). However, the change was not associated with a reduced SAM/SAH ratio but rather only a decrease in SAM. Choline levels can only be accurately measured in microwave radiated mice and indeed brain tissue levels were decreased in the mid-brain.

Liver, kidney, frontal cortex and cortex brain tissue concentrations of ADMA and SDMA were increased in young folate deficient mice sacrificed by CO_2 asphyxiation (Tables 19 and 21-22). In contrast, only cortical levels were altered in old mice sacrificed by CO_2 asphyxiation (Table 24). Although decreased SAM/SAH ratio is indicative of hypomethylation it is likely to be tissue and methyltransferase specific (James et al. 2002). Also high concentrations of Hcy have been shown to be an inhibitor of DDAH in mice (Tyagi et al. 2005; Stuhlinger and Stanger 2005; Dayal et al. 2008), thereby, increasing ADMA levels via decreased catabolism. However, data presented in

this chapter does not support the notion that folate deficiency and elevated tHcy can influence ADMA and SDMA concentrations since no changes were observed in folate deficient mice sacrificed by microwave radiation (Tables 26 and 27). Thus, the alterations of ADMA and SDMA in folate deficient mice sacrificed by CO_2 asphyxiation are presumably due to post-mortem events.

A limitation of this study is that folate concentrations in brain tissue were not measured, due to insufficient amount of brain tissue for this analysis. The assessment of folate deficiency is based primarily on decreased plasma 5-MTHF and elevated plasma tHcy. It is therefore uncertain to what extent folate levels have been affected in regional brain tissues. Varela-Moreiras and Selhub reported that rats fed a folate deficient diet for 25 weeks were able to maintain 5-MTHF levels in liver tissue but total folate was reduced 60% (Varela-Moreiras and Selhub 1992). They also did not observe any changes in total folate or 5-MTHF in brain tissue from folate deficient rats. Similarly, 3 week old MTR heterozygous mice fed a low folate diet for 7 to 17 weeks also had significantly reduced total folate levels in liver, but not in brain tissue (Dayal et al. 2005). These studies suggest that the rodent brain may be partially resistant to folate depletion by dietary folate deficiency at the expense of the liver. The capability of the brain to conserve folate may be due to a highly effective mechanism of folate transport across the BBB. However, in the former study SSA was not included in the diet and rats were studied, thus folate deficiency was not as severe as in this study. Also in the latter study brain folate levels did decrease but not to a significant degree but it is plausible that a longer treatment period would have been sufficient to induce changes in brain folate levels. The methylation data presented in this chapter suggests that both liver and brain

folate levels may have been altered due to dietary folate restriction, although the extent of the deficient was not assessed.

tg-MTHFR Aged Low Folate Study

Effect of gender and genotype. No differences in plasma 5-MTHF or tHcy were found due to gender in the *tg*-MTHFR mice (Table 29). Chen and co-workers also reported similar findings in their study of *tg*-MTHFR mice (Chen et al. 2001). Methionine levels were elevated in brain tissues of female *tg*-MTHFR mice compared to males of the same genotype (Table 29). Recently, it was reported that transmethylation and remethylation rates are higher in women possibly due to hormonal differences (Fukagawa et al. 2000). The data present here suggests this may also be the case in female mice.

Plasma 5-MTHF levels were significantly decreased in heterozygous males compared to wild type mice (Table 30). Plasma tHcy levels were dependent on the MTHFR genotype, demonstrating that even MTHFR heterozygous mice, with as much as 60% residual MTHFR activity, were not able to maintain Hcy homeostasis in the presence of adequate folate. This finding has previously been reported in several other mouse and human studies (Knock et al. 2008; Schwahn et al. 2003; Li et al. 2005).

Heterozygous *tg*-MTHFR mice have a decreased SAM/SAH ratio caused by decreased SAM concentrations in liver, but not in brain tissues (Tables 31-33). This data is consistent with previous studies, which also reported genotype related changes in liver tissues for SAM and the SAM/SAH ratio in *tg*-MTHFR mice (Delvin et al. 2004, Schwahn et al. 2004; Chen et al. 2001). Additionally, Delvin and colleagues reported

that the decreased SAM/SAH ratio was not associated with global DNA hypomethylation in the liver. Liver betaine levels were decreased in heterozygotes presumably due to increased demand as a methyl donor since 5-MTHF levels are potentially decreased in liver (Table 31). The data presented here is in accordance with Chen and co-workers, who reported that total folate is maintained in heterozygous *tg*-MTHFR mice in liver and brain tissues but the percentage of 5-MTHF is significantly decreased in the liver but not brain tissues (Chen et al. 2001).

Increased cystathionine levels were observed in the frontal cortex, cortex, cerebellum, and mid-brain of heterozygous *tg*-MTHFR mice due to increased influx into the transsulfuration pathway resulting from increased concentrations of Hcy (Tables 32 and 33). Furthermore, cystathionine becomes trapped due to the absence of CSL activity in brain tissue (Ishill et al. 2004). No alterations of cystathionine were present in liver, as may be expected since CSL is active in this organ allowing metabolism through to the transsulfuration pathway leading to the synthesis of cysteine and glutathione (Table 31). The data presented between the two genotypes of the *tg*-MTHFR mouse model suggests that a mild elevation of Hcy is not sufficient to modify the methylation cycle although; heterozygote mice do have elevated plasma tHcy compared to wild type mice.

Effect of low folate. Low folate diet for 6 months in *tg*-MTHFR mice caused mild to moderate HHcy in wild type, which were exacerbated in the heterozygous genotype (Table 30). Thus, reduced MTHFR enzyme activity in heterozygous *tg*-MTHFR acts synergistically with dietary folate deficiency to alter plasma tHcy levels in mice. The change in other methylation metabolites of wild type and heterozygous *tg*-MTHFR mice fed a low folate diet for 6 months were the same as the changes reported in young

C57BL/6J mice fed a folate deficient diet sacrificed by CO_2 asphyxiation in brain tissues (decreased SAM, SAM/SAH, methionine, and betaine with increased SAH and cystathionine) (Tables 32 and 33). Furthermore, plasma and liver tissues levels were also affected in this mouse model (Tables 30 and 331). Surprisingly, the decreased SAM or increased SAH caused by low folate status were not worsened in heterozygous mice compared to wild type mice. In 2004 Devlin and co-workers published a low folate study in 3 week old *tg*-MTHFR with a 7 to 15 week treatment period and found almost identical finding in regards to SAM, SAH and the ratio in brain and liver tissues (Devlin et al. 2004). Interesting, a low folate diet exacerbated the increase in cystathionine observed in all brain regions in *tg*-MTHFR heterozygous mice compared to wild type on the same diet (Tables 32 and 323). This may indicate increased cellular production of Hcy and conversion through to cystathionine in CNS tissue of *tg*-MTHFR heterozygous mice with low folate. As previously mentioned the significance of elevated cystathionine in brain tissue is not known. This may have important implications in CNS disorders since the MTHFR 677TT genotype is prevalent in up to 12 - 22% of human populations of which some may be more severely affected by a low folate status (Brotto and Yang 2000).

The extent of change of the methylation metabolites investigated was very similar in aged *tg*-MTHFR mice fed a low folate diet and young C57BL/6J mice fed a folate deficient diet. This observation is somewhat surprising since *tg*-MTHFR mice were older, fed treatment diet 3 months longer, and have an abnormality in an enzyme that affects the methylation cycle, although their treatment diet did have higher folate content. The use of the heterozygous *tg*-MTHFR mouse model may remain important due to its

similarity to the common human MTHFR C677T polymorphism. It is also important to note that this is the second study using an aged *tg*-MTHFR model to study methylation. The other study investigated the effects of the MTHFR mutation on intestinal tumorigenesis; however, metabolites of the methylation cycle were not measured (Knock et al. 2008). Therefore, more studies are needed to determine the effect of decreased MTHFR activity in an aged mouse model on CNS function.

Summary

This is the first study investigating a comprehensive panel of methylation metabolites in peripheral and CNS tissues in aged folate deficient mouse models. The additional studies explore differences with the use of microwave radiation as the method of sacrificing mice. Surprisingly, an increase in age did not exacerbate the effects of folate deficiency with regard to changes in methylation metabolites nor did a MTHFR heterozygous genotype. The folate deficient related changes in the methylation metabolites, specifically, decreased SAM/SAH ratio reported here validated previous reports on the effects of folate deficiency. The use of microwave fixation as a rapid killing method revealed that reduced SAM levels rather than increased SAH levels is responsible for the lower SAM/SAH ratio that reflect methylation activity. Other significant changes that require further study include the increased accumulation of cystathionine in brain tissue from *tg*-MTHFR heterozygous mice on a low folate diet. The following chapters aim to establish the consequence(s) of folate deficiency and the MTHFR genotype on brain neurotransmitters and cognitive function.

CHAPTER FIVE

The Effect of Folate Deficiency on Neurotransmitter Metabolism in Young C57BL/6J Mice Sacrificed by Microwave Radiation

Introduction

Deficiencies in several neurotransmitter systems have been implicated in a wide variety of psychiatric and neurological disorders. Evidence suggests that the folate enzymes, DHFR and MTHFR, are involved in salvage pathways for regeneration of BH_4, the rate-limiting cofactor for the biosynthesis of dopamine, norepinephrine, and serotonin (Kaufman 1981; Kaufman 1991). Furthermore, folate is essential as a starting material for pterin synthesis (Anderson et al. 1992). BH_4 also acts on membrane receptors to directly stimulate release of neurotransmitters presumably by activating ion channels, and is thought to indirectly stimulate the release of non-monoamine neurotransmitters by activation of monoaminergic systems (Kaufman et al. 1991).

It has been shown that up to one third of severely depressed patients are folate deficient and this is accompanied by a reduction of CSF 5-HIAA, the main metabolite of serotonin (Carney et al. 1990; Godfrey et al. 1990; Bottiglieri et al. 1992; Bottiglieri et al. 2000). In addition, the MTHFR C677T genotype has been shown in some studies to be over represented in depressed patients lending further support to the implication of the involvement of folate in neurotransmitter synthesis (Kelly et al. 2004; Gilbody et al. 2007). Also children with a severe in-born error of folate metabolism (MTHFR deficiency) have decreased levels of BH_4, HVA, and 5-HIAA (metabolites of dopamine and serotonin, respectively) in CSF (Kaufman 1981; Surtees et al. 1994). Further support

for the role of folate in monoamine neurotransmitter metabolism is evident in a study showing that intra-cerebroventricular injections of the antifolate drug, methotrexate, reduced the concentrations of dopamine, serotonin, and NE in rat brain tissue (Madhyastha et al. 2002).

Three other neurotransmitters, glutamate, GABA, and acetylcholine may also be linked to folate metabolism. Folate is polyglutamated; thereby glutamate may be altered during instances of inadequate folate. Since glutamate is a precursor to GABA this neurotransmitter may also be affected. Another neurotransmitter system which may be influenced by folate status is acetylcholine. Folate metabolism provides methyl groups derived from SAM to form phosphatidylcholine that is a source of choline. As described in detail in chapter 1, choline and acetyl CoA are substrates for choline acetyltransferase (ChAT) in the production of acetylcholine. Folate deficiency has been shown to reduce the phosphatidylcholine content in rat brain tissue which has been proposed, but not proven, to decrease choline and acetylcholine pools (Troen et al. 2008). It has been reported that the dopaminergic and cholinergic neurotransmitter systems undergo age-related neuronal degeneration (Freeman and Gibson 1988; Kay 1986). Excitotoxicity due to increased glutamate is involved in several neurodegenerative disorders (Hynd et al. 2004).

Owing to a substantial number of reports linking low folate levels with altered monoamine neurotransmitters levels mainly from studies performed in humans, it is essential to replicate this association in an animal model in order to study the mechanism(s) involved. Since the early 1970s it has been established that many neurotransmitter systems are sensitive to post-mortem changes and therefore accurate

quantitation can only be accomplished in animals sacrificed by microwave radiation (Stavinoha et al. 1973; Groppetti et al. 1977; Moroji et al. 1977). To date, only a few *in vivo* mouse studies have been performed that examined the role of folate in the regulation of neurotransmitter metabolism and these have not used microwave radiation as the method of sacrificing animals (Gospe et al. 1995). Studies presented in this chapter aim to clarify and give a more comprehensive view of several neurotransmitter systems in folate deficient mice that have been sacrificed by microwave radiation.

Results

Effect of Microwave Radiation

Tables 35, 36, and 37 outline the regional brain tissue concentrations of the various neurotransmitters and their metabolites studied in this work from young mice sacrificed by CO_2 asphyxiation and microwave radiation. The data obtained for dopamine and its metabolites were significantly different between the two methods of sacrifice. Dopamine was significantly elevated in four of the six regions analyzed from mice sacrificed by microwave radiation compared to mice sacrificed by CO_2 asphyxiation. In these same brain regions DOPAC and 3-MT were decreased due to microwave radiation. However, 3-MT levels were increased in the hippocampus from mice sacrificed by microwave radiation compared to those sacrificed by CO_2 asphyxiation. Brain tissue concentrations of HVA were increased in the striatum and decreased in the frontal cortex in mice sacrificed by microwave radiation. Intra-neuronal dopamine turnover (DOPAC+HVA/DA ratio) was reduced in the striatum, hippocampus, frontal cortex, and cortex, and extra-neuronal turnover (3-MT/DA ratio), in the striatum,

frontal cortex, cortex, and mid-brain in mice sacrificed by microwave radiation. In addition, an increase in extra-neuronal dopamine turnover was detected in the hippocampus from mice sacrificed by microwave radiation. Tyrosine was not affected by method of sacrifice.

Table 35. Brain tissue (striatum and hippocampus) neurotransmitters and their metabolites: effect of microwave radiation.

Metabolite	CO_2 Control Diet	MW Control Diet
	STRIATUM	
BH_4	1.4 ± 0.1	0.92 ± 0.17*
DA	74.1 ± 21.2	73.7 ± 21.4
DOPAC	20.3 ± 2.5	0.02 ± 0.01*
HVA	13.1 ± 1.6	8.3 ± 1.9*
3-MT	6.7 ± 0.7	0.60 ± 0.18*
DOPAC+HVA/DA	0.48 ± 0.14	0.12 ± 0.02*
3-MT/DA	0.10 ± 0.02	0.01 ± 0.001*
5-HT	5.3 ± 1.4	12.4 ± 2.0*
5-HIAA	4.5 ± 0.9	5.2 ± 0.9
5-HIAA/5-HT	0.87 ± 0.12	0.42 ± 0.06*
NE	0.81 ± 0.30	0.65 ± 0.20*
	HIPPOCAMPUS	
BH_4	0.28 ± 0.09	0.28 ± 0.04
DA	0.50 ± 0.16	0.94 ± 0.21*
DOPAC	0.67 ± 0.16	0.02 ± 0.01*
HVA	0.40 ± 0.08	0.42 ± 0.08
3-MT	0.09 ± 0.05	0.33 ± 0.09*
DOPAC+HVA/DA	2.0 ± 0.7	0.49 ± 0.12*
3-MT/DA	0.14 ± 0.06	0.35 ± 0.07*
5-HT	7.4 ± 2.5	13.9 ± 1.8*
5-HIAA	7.8 ± 1.7	6.3 ± 1.3
5-HIAA/5-HT	1.1 ± 0.2	0.45 ± 0.06*
NE	3.7 ± 1.1	2.3 ± 0.5*

nmol/g, Values expressed as mean ± SD (n=6-8)

* $p < 0.05$ Compared to CO_2 of same region

Serotonin levels were significantly increased in all six brain regions studied due to microwave radiation. Microwave radiation also caused an increase in 5-HIAA, in the

cortex and cerebellum. Serotonin turnover (5-HT/5-HIAA ratio), was decreased in all brain regions analyzed from mice sacrificed by microwave radiation compared to mice sacrificed by CO_2 asphyxiation. Additionally, tryptophan levels were increased in the cortex from mice sacrificed by microwave radiation compared to mice sacrificed by CO_2 asphyxiation.

Table 36. Brain tissue (frontal cortex and cortex) neurotransmitters and their metabolites: effect of microwave radiation.

Metabolite	CO2 Control Diet	MW Control Diet
	FRONTAL CORTEX	
DA	3.5 ± 2.8	18.8 ± 8.2*
DOPAC	3.1 ± 1.8	0.07 ± 0.02*
HVA	1.9 ± 0.8	3.2 ± 1.1*
3-MT	0.41 ± 0.24	0.14 ± 0.07*
DOPAC+HVA/DA	1.8 ± 1.1	0.18 ± 0.04*
3-MT/DA	0.14 ± 0.07	0.01 ± 0.002*
5-HT	4.7 ± 1.8	17.1 ± 1.5*
5-HIAA	4.6 ± 1.5	4.2 ± 0.6
5-HIAA/5-HT	1.2 ± 0.7	0.25 ± 0.05*
NE	3.0 ± 0.6	1.9 ± 1.2
	CORTEX	
BH_4	0.36 ± 0.05	0.38 ± 0.07
TYR	79.7 ± 14.1	92.8 ± 25.9
DA	3.9 ± 1.1	10.5 ± 3.6*
DOPAC	1.3 ± 0.3	0.06 ± 0.04*
HVA	1.5 ± 0.3	1.9 ± 0.6
3-MT	0.55 ± 0.11	0.10 ± 0.05*
DOPAC+HVA/DA	0.74 ± 0.13	0.21 ± 0.04*
3-MT/DA	0.15 ± 0.04	0.01 ± 0.01*
TRP	19.7 ± 9.9	31.4 ± 9.3*
5-HT	5.5 ± 0.8	14.9 ± 1.2*
5-HIAA	2.5 ± 0.4	3.2 ± 0.6*
5-HIAA/5-HT	0.44 ± 0.05	0.21 ± 0.03*
NE	2.8 ± 0.3	6.3 ± 1.4*
GABA	1740 ± 141	1586 ± 248
GLU	7619 ± 596	9467 ± 1604*
GLN	3421 ± 233	4611 ± 383*
Ach	3.6 ± 1.0	39.4 ± 10.3*

nmol/g, Values expressed as mean ± SD (n=6-8)

* $p < 0.05$ Compared to CO_2 of same region

Table 37. Brain tissue (cerebellum and mid-brain) neurotransmitters and their metabolites: effect of microwave radiation.

Metabolite	CO2 Control Diet	MW Control Diet
	CEREBELLUM	
BH_4	0.31 ± 0.03	0.29 ± 0.04
TYR	87.5 ± 19.7	96.8 ± 16.0
DA	0.16 ± 0.04	0.24 ± 0.09*
DOPAC	0.17 ± 0.02	0.13 ± 0.09
HVA	0.11 ± 0.05	0.18 ± 0.06
3-MT	0.02 ± 0.01	n/d
DOPAC+HVA/DA	1.8 ± 0.5	1.5 ± 0.8
3-MT/DA	0.11 ± 0.08	n/a
TRP	17.6 ± 2.2	30.7 ± 4.0*
5-HT	2.0 ± 0.7	8.2 ± 1.9*
5-HIAA	0.91 ± 0.21	1.9 ± 0.9*
5-HIAA/5-HT	0.47 ± 0.10	0.22 ± 0.05*
NE	2.9 ± 0.6	2.1 ± 0.4
GABA	2029 ± 197	1336 ± 57*
GLU	7964 ± 1479	8342 ± 552
GLN	5449 ± 538	4401 ± 225
Ach	9.4 ± 3.6	15.6 ± 4.7*
	MID-BRAIN	
BH_4	0.58 ± 0.05	0.58 ± 0.05
TYR	70.1 ± 25.9	86.8 ± 11.7
DA	1.3 ± 0.4	1.6 ± 0.3
DOPAC	0.78 ± 0.17	0.81 ± 1.19
HVA	1.1 ± 0.2	1.2 ± 0.2
3-MT	0.07 ± 0.04	0.01 ± 0.01*
DOPAC+HVA/DA	1.5 ± 0.3	1.4 ± 1.1
3-MT/DA	0.05 ± 0.02	0.01 ± 0.004*
TRP	23.8 ± 5.7	28.3 ± 3.0
5-HT	6.1 ± 1.2	21.1 ± 2.0*
5-HIAA	6.3 ± 0.6	7.2 ± 1.5
5-HIAA/5-HT	1.1 ± 0.4	0.34 ± 0.04*
NE	3.3 ± 1.3	3.0 ± 0.4
GABA	2930 ± 867	2770 ± 273
GLU	5725 ± 1562	8042 ± 231*
GLN	3863 ± 1104	4095 ± 371
Ach	2.7 ± 1.1	53.3 ± 2.9*

nmol/g, Values expressed as mean ± SD (n=6-8)

* $p < 0.05$ Compared to CO_2 of same region

n/d= none detected; n/a= not applicable

Microwave radiation caused a reduction in norepinephrine in five brain regions, but was only statistically significant in the striatum and hippocampus. However, norepinephrine levels were increased in the cortex from mice exposed to microwave radiation. The essential cofactor for the biosynthesis of all three of these neurotransmitters, BH_4, was not greatly affected by microwave radiation, the only changes observed was lower levels in the striatum.

Glutamate was also affected by sacrifice method. Mice sacrificed by microwave radiation had increased levels in the cortex and mid-brain. Additionally, microwave radiation resulted in elevated cortical glutamine concentrations. Brain levels of GABA were unaffected by the methods of sacrifice. However, acetylcholine was significantly increased in all three regions studied from mice sacrificed by microwave radiation compared to CO_2 asphyxiated mice.

Effect of Folate Deficiency

The effect of folate deficiency on various neurotransmitters and their corresponding metabolites in young mice sacrificed by microwave radiation is presented in tables 38, 39, and 40. Rearing on a low folate diet did not generally impact any of the neurotransmitter systems studies, however, rearing on a folate deficient diet did. Dopamine was elevated only in the hippocampus and the metabolites, DOPAC and 3-MT were not altered in any brain regions from mice fed a folate deficient diet compared to mice fed a control diet. However, HVA was decreased in the striatum and cortex from young mice fed a folate deficient diet compared to those fed a control diet. More importantly, a folate deficient diet decreased intra- and extra-neuronal dopamine turnover

Table 38. Brain tissue (striatum and hippocampus) neurotransmitters and their metabolites from young mice sacrificed by microwave radiation: effect of folate.

Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
		STRIATUM	
BH_4	0.92 ± 0.17	0.88 ± 0.09	0.87 ± 0.04
TYR	95.5 ± 17.0	82.1 ± 39.3	85.1 ± 19.5
DA	73.7 ± 21.4	78.8 ± 10.0	85.1 ± 10.9
DOPAC	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.02
HVA	8.3 ± 1.9	8.1 ± 1.0	5.8 ± 0.4*
3-MT	0.60 ± 0.18	0.79 ± 0.11	0.57 ± 0.08
DOPAC+HVA/DA	0.12 ± 0.02	0.10 ± 0.01	0.07 ± 0.01*
3-MT/DA	0.008 ± 0.001	0.010 ± 0.002*	0.007 ± 0.001*
TRP	43.4 ± 8.8	36.7 ± 8.2	35.4 ± 10.6
5-HT	12.4 ± 2.0	11.6 ± 0.88	11.1 ± 1.6
5-HIAA	5.2 ± 0.9	4.7 ± 0.9	3.4 ± 0.3*
5-HIAA/5-HT	0.42 ± 0.06	0.41 ± 0.07	0.31 ± 0.03*
NE	0.65 ± 0.20	0.58 ± 0.06	0.65 ± 0.10
GABA	2203 ± 416	1862 ± 313	1722 ± 204
GLU	9837 ± 968	10066 ± 962	8536 ± 551*
GLN	6327 ± 646	5788 ± 249	6002 ± 356
ACH	122.3 ± 15.4	111.4 ± 7.23	110.7 ± 11.8
		HIPPOCAMPUS	
BH_4	0.28 ± 0.04	0.28 ± 0.05	0.27 ± 0.04
TYR	90.9 ± 18.3	73.7 ± 32.8	81.2 ± 17.4
DA	0.94 ± 0.21	1.0 ± 0.4	2.7 ± 1.2*
DOPAC	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
HVA	0.42 ± 0.08	0.38 ± 0.10	0.39 ± 0.08
3-MT	0.33 ± 0.09	0.35 ± 0.08	0.36 ± 0.07
DOPAC+HVA/DA	0.49 ± 0.12	0.41 ± 0.08	0.18 ± 0.08*
3-MT/DA	0.35 ± 0.07	0.37 ± 0.11	0.17 ± 0.11*
TRP	29.5 ± 5.9	20.5 ± 6.9	23.3 ± 8.4
5-HT	13.9 ± 1.8	12.8 ± 2.4	13.2 ± 0.8
5-HIAA	6.3 ± 1.3	5.4 ± 1.0	4.0 ± 0.5*
5-HIAA/5-HT	0.45 ± 0.06	0.43 ± 0.11	0.30 ± 0.03*
NE	2.3 ± 0.5	2.0 ± 0.7	2.0 ± 0.3
GABA	1808 ± 200	1534 ± 411	1413 ± 134*
GLU	10155 ± 850	9015 ± 1090	8425 ± 906*
GLN	5802 ± 635	4773 ± 590*	5037 ± 606
ACH	39.6 ± 4.6	32.0 ± 9.3	34.5 ± 4.2

nmol/g, Values expressed as mean ± SD (n=5-6)

* $p < 0.05$ Compared to CD of same region

Table 39. Brain tissue (cortex) neurotransmitters and their metabolites from young mice sacrificed by microwave radiation: effect of folate.

Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
		CORTEX	
BH_4	0.38 ± 0.07	0.41 ± 0.04	0.33 ± 0.07
TYR	92.8 ± 25.9	84.9 ± 32.8	86.6 ± 21.2
DA	10.5 ± 3.6	12.6 ± 2.6	9.6 ± 3.1
DOPAC	0.06 ± 0.04	0.05 ± 0.04	0.05 ± 0.02
HVA	1.9 ± 0.6	2.0 ± 0.1	1.2 ± 0.2*
3-MT	0.10 ± 0.05	0.10 ± 0.04	0.11 ± 0.03
DOPAC+HVA/DA	0.21 ± 0.04	0.17 ± 0.04	0.14 ± 0.03*
3-MT/DA	0.011 ± 0.001	0.008 ± 0.002	0.012 ± 0.004
TRP	31.4 ± 9.3	25.6 ± 6.9	24.9 ± 5.5
5-HT	14.9 ± 1.2	15.5 ± 1.6	15.9 ± 1.8
5-HIAA	3.2 ± 0.6	2.8 ± 0.5	2.3 ± 0.4*
5-HIAA/5-HT	0.21 ± 0.03	0.18 ± 0.03	0.15 ± 0.02*
NE	6.3 ± 1.4	5.9 ± 1.7	6.8 ± 1.1
GABA	1586 ± 248	1669 ± 245	1648 ± 350
GLU	9467 ± 1604	9400 ± 948	8740 ± 290
GLN	4611 ± 383	4278 ± 676	4635 ± 500
ACH	39.4 ± 10.3	39.4 ± 5.2	35.9 ± 3.2

nmol/g, Values expressed as mean ± SD (n=5-6)
* $p < 0.05$ Compared to CD of same region

in the striatum and hippocampus. In addition, extra-neuronal dopamine turnover was also decreased in the cortex from mice fed a folate deficient diet compared to mice fed a control diet. Folate deficiency had no effect on, tryptophan or serotonin levels in brain tissues from mice sacrificed by microwave radiation. The concentration of 5-HIAA was decreased in four brain regions (striatum, hippocampus, cortex, and mid-brain) from mice fed a folate deficient diet. This resulted in significantly decreased serotonin turnover (5-HIAA/5-HT) in those same regions.

Brain tissue levels of norepinephrine and BH_4 were not altered by folate deficiency. Three other neurotransmitters, GABA, glutamate, and acetylcholine were affected by dietary folate restriction. Rearing on a folate deficient diet resulted in

Table 40. Brain tissue (cerebellum and mid-brain) neurotransmitters and their metabolites from young mice sacrificed by microwave radiation: effect of folate.

Metabolite	Control Diet	Low Folate Diet	Folate Deficient Diet
		CEREBELLUM	
BH_4	0.29 ± 0.04	0.33 ± 0.02	0.28 ± 0.02
TYR	96.8 ± 16.0	90.6 ± 37.2	89.7 ± 18.9
DA	0.24 ± 0.09	0.33 ± 0.21	0.27 ± 0.05
DOPAC	0.13 ± 0.09	0.17 ± 0.04	0.14 ± 0.04
HVA	0.18 ± 0.06	0.11 ± 0.01	0.17 ± 0.03
3-MT	n/d	0.17 ± 0.03	0.11 ± 0.01
DOPAC+HVA/DA	1.5 ± 0.8	0.03 ± 0.01	0.02 ± 0.01
3-MT/DA	n/a	1.3 ± 0.6	0.93 ± 0.09
TRP	30.7 ± 4.0	27.2 ± 6.9	25.3 ± 5.3
5-HT	8.2 ± 1.9	7.7 ± 0.9	7.5 ± 1.1
5-HIAA	1.9 ± 0.9	1.3 ± 0.2	1.1 ± 0.2
5-HIAA/5-HT	0.22 ± 0.05	0.17 ± 0.03	0.14 ± 0.02*
NE	2.1 ± 0.4	2.2 ± 0.1	2.1 ± 0.2
GABA	1336 ± 57	1320 ± 106	1269 ± 123
GLU	8342 ± 552	8108 ± 898	7560 ± 444
GLN	4401 ± 225	4213 ± 273	4050 ± 401
ACH	15.6 ± 4.7	13.7 ± 5.2	12.7 ± 1.3
		MID-BRAIN	
BH_4	0.58 ± 0.05	0.63 ± 0.02	0.56 ± 0.05
TYR	86.8 ± 11.7	90.4 ± 37.3	78.8 ± 29.9
DA	1.6 ± 0.3	3.7 ± 2.5	3.2 ± 2.4
DOPAC	0.81 ± 1.19	0.29 ± 0.13	0.27 ± 0.06
HVA	1.2 ± 0.2	1.4 ± 0.4	1.0 ± 0.3
3-MT	0.014 ± 0.006	0.019 ± 0.010	0.014 ± 0.005
DOPAC+HVA/DA	1.4 ± 1.1	0.59 ± 0.29	0.51 ± 0.18
3-MT/DA	0.009 ± 0.004	0.006 ± 0.002	0.005 ± 0.002
TRP	28.3 ± 3.0	27.0 ± 4.0	20.6 ± 8.2
5-HT	21.1 ± 2.0	20.7 ± 1.1	21.1 ± 1.9
5-HIAA	7.2 ± 1.5	5.9 ± 0.9	5.0 ± 0.6*
5-HIAA/5-HT	0.34 ± 0.04	0.29 ± 0.04	0.23 ± 0.02*
NE	3.0 ± 0.4	3.2 ± 0.3	3.2 ± 0.3
GABA	2770 ± 273	2983 ± 141	3168 ± 662
GLU	8042 ± 231	7888 ± 354	7000 ± 1141
GLN	4095 ± 371	3925 ± 190	3770 ± 599
ACH	53.3 ± 2.9	55.9 ± 3.8	45.6 ± 5.2*

nmol/g, Values expressed as mean ± SD (n=4-6)
* $p < 0.05$ Compared to CD of same region
n/d= not detected, n/a= not applicable

reduced hippocampal GABA and glutamate levels; glutamate was also reduced in the striatum. Acetylcholine was also reduced in young mice fed folate deplete diet however only significantly in the mid-brain. Table 41 summarizes all the changes attributed to folate deficiency in young mice sacrificed by microwave radiation.

Table 41. Brain tissue neurotransmitters and their metabolites summary from young mice sacrificed by microwave radiation: effect of folate.

	The Effect of Folate Deficiency
Metabolite	Young
BH_4	none
TYR	none
DA	HIP ↑
DOPAC	none
HVA	STR,CX ↓
3-MT	none
DOPAC+HVA/DA	STR,HIP,CX ↓
3-MT/DA	STR,HIP ↓
TRP	none
5-HT	none
5-HIAA	STR,HIP,CX,MB ↓
5-HIAA/5-HT	STR,HIP,CX,CB,MB ↓
NE	none
GABA	HIP ↓
GLU	STR,HIP ↓
GLN	none
ACH	MB ↓

Discussion

Effect of Microwave Radiation

It has been known since the 1970s that there are vast differences in brain neurotransmitters and related metabolites in mice and rats sacrificed by CO_2 asphyxiation versus those sacrificed by focused microwave radiation. This work further investigates these differences by looking at a greater number of metabolites than previously reported; in addition, regional specific metabolite concentrations were measured.

It has been suggested that microwave radiation causes disruption of cellular membranes causing diffusion of substances and this is the reason why microwaved mice have different concentrations of neurotransmitters than CO_2 asphyxiated mice (Sharpless et al. 1978). In 1982 Ishikawa and co-workers proved this theory false by showing that the differences are due to the speed of inhibition of enzyme activity (Ishikawa et al. 1982). Indeed considerable significant differences were seen in all of the neurotransmitters studied except for GABA. The monoamines, serotonin and dopamine were markedly increased in all brain regions after microwave radiation compared to CO_2 asphyxiated mice (Tables 35-37). Heat inactivation of the enzymes responsible for their catabolism are suspected to be the cause, hence estimates of turnover are decreased, this concurs with the fact that dopamine decreases post-mortem and its metabolites increase. Many other studies have shown this same finding in a less extensive manner (Groppetti et al. 1977, Moroji et al. 1977; Ishikawa et al. 1982). The exact values were comparable to those reported in the literature (Ishikawa et al. 1982; Bertrand et al. 1994). However, one group did report that microwave does not affect the parent neurotransmitters to any great extent (Maruyama et al. 1980) and much of the quantitative studies on neurotransmitters in the literature today do not involve the use of microwave radiation (Chan et al. 2008; Lee et al. 2005; Diaz-Ruiz et al. 2009). Norepinephrine levels were decreased as expected, possibly due to its precursor dopamine not being metabolized by dopamine β-hydroxylase (Tables 35-37). Lastly, acetylcholine levels were highly sensitive to method of sacrifice, which is in agreement with previous reports (Tables 35-37). It has been shown that acetylcholine levels increase and choline levels decrease after exposure to microwave radiation for 1 second due to complete inactivation of acetylcholine esterase

and choline acetyltransferase (Butcher et al. 1976; Bertrand et al. 1994). Thus, in order to obtain a meaningful result for brain acetylcholine, animals must be sacrificed by the use of a microwave radiation system to eliminate post-mortem changes (Moroji et al. 1997; Schmidt et al. 1972; Stavinoha et al. 1973).

Effect of Folate Deficiency

In this study it could not be confirmed that dietary folate deficiency affects BH_4 concentrations in the mouse brain (Tables 38-40). This finding does not support previous observations that folate is required in maintaining BH_4 concentrations through a secondary salvage pathway involving the regeneration of qBH_2 to BH_4 in mouse brain tissue (see Figure 5, Chapter 1). Consequently, no deficits in brain levels of dopamine, serotonin, or norepinephrine were found in folate deficient mice, indicating that the synthesis pathway for these neurotransmitters is not affected (Tables 38-40). As previously mentioned (chapter 4) it is uncertain to what extent folate deficiency occurred in brain tissue. Thereby, it is possible that mice reared on a folate deficient diet for longer than 3 months may eventually have an effect on BH_4 and monoamine neurotransmitter synthesis. It appears that the mouse brain is more resistant to dietary folate deficiency than humans, because even a short insufficiency of folate in humans leads to alterations of neurotransmitters.

In a previous study it has been reported that dopamine and HVA levels were significantly reduced in the caudate brain region of mice fed a folate deficient diet for 37 days (Gospe et al. 1995). Interestingly, in most brain regions there was a decrease in the DOPAC+HVA/DA ratio, an indicator of dopamine turnover (Table 38-40). It is unlikely that dopamine release is affected since there was no significant change in the

concentration of 3-MT, a metabolite that is only formed when dopamine is released into the synaptic cleft. The reduced turnover of dopamine in folate deficient mice is therefore likely to originate from intra-neuronal metabolism, which is confirmed by the decreased levels of DOPAC. Likewise, a decrease in the 5-HIAA/5-HT ratio in all brain regions from folate deficient mice is indicative of a reduced serotonin turnover rate (Tables 38-40). It is difficult to assess if serotonin release is affected since the concentration of serotonin in the synaptic cleft is a fraction of the intra-neuronal pool. Further studies using *in vivo* microdialysis that can measure interstitial serotonin concentrations would be better suited to determine the effect of folate deficiency on serotonin release. Other investigators have also shown that folate deficiency has a predominant effect on brain serotonin metabolism with lower 5-HIIAA levels reported in rat brain (Botez et al. 1979) and in the hypothalamus of the mouse brain (Gospe et al. 1995). In the latter study this was associated with an increase in food spilling indicating a deficit in coordination as a result of dopaminergic and/or serotonergic imbalance.

The reduced turnover rates for dopamine and serotonin observed in folate deficient mice in this study are consistent with observations in children with severe MTHFR deficiency that were reported to have reduced levels of HVA and 5-HIAA in CSF (Hyland et al. 1988). Reduced levels of 5-HIAA and HVA in CSF have been also been reported in folate deficient adults with epilepsy and depression (Botez et al. 1979; Bottiglieri et al. 2000). Reduced serotonin turnover may affect the functional activity of serotonergic pathways throughout various brain regions. This neurochemical imbalance may account for the increase in depressive symptoms in folate deficient patients, since

aberrant serotonin metabolism has been implicated in the neurobiology of major depressive disorder (Nordquist and Oreland 2010).

The inhibitory amino acid neurotransmitter, GABA, was also affected by folate deficiency in some brain regions (Tables 38-40). The precursor amino acid glutamate was significantly decreased in the hippocampus and striatum, accompanied by a 22% reduction in GABA in both regions, which was only statistically significant in the hippocampus. Related to this, other investigators have shown that dietary folate deficiency did not affect glutamate decarboxylase or gamma-amino-butyrate aminotransferase in brain tissue from rats fed a folate deficient diet for 6 weeks (Botez et al. 1980). The effect of folate deficiency on brain glutamate and GABA concentrations has not been previously studied in either rodent models or in human CSF. However, in humans, depressed patients have lower plasma, CSF and brain GABA concentrations than non-depressed subjects (Sanacora and Saricicek 2007). The role of GABA in depression is reinforced by evidence that GABA-mimetic drugs have antidepressant properties (Cryan and Kaupmann 2005). Although the mechanism that causes reduced brain GABA levels in folate deficiency is not clear, this may potentially be a neurochemical pathway involved in folate deficient subjects with depression.

The effect of folate deficiency on the neurotransmitter acetylcholine has been studied in this dissertation. The data presented showed a significant reduction (15%) in the mid-brain region only in mice fed a folate deficient diet compared to mice fed a control diet (Table 40). This was associated with a significant reduction in the concentration of choline in this brain region, lending support to the hypothesis that inhibition of methylation of phosphatidylethanolamine to phosphatidylcholine occurs in

folate deficiency, leading to reduced choline and acetylcholine pools in the brain. In regions other than the mid-brain choline and acetylcholine levels tended to be lower but were not statistically significant. Reduced methylation of phosphatidylethanolamine to phosphatidylcholine has been demonstrated in brain tissue from rats fed a folate deficient diet for 10 weeks (Troen et al. 2008). However these investigators were not able to determine brain acetylcholine since the animals were sacrificed by decapitation and not by focused microwave radiation. In addition, these investigators showed that supplementation of the folate deficient diet with methionine could reverse the inhibition of phosphatidylethanolamine methylation and normalize phosphatidylcholine levels (Troen et al. 2008). Collectively these studies suggest that methylation of phospholipids can be modulated by dietary folate and methionine and affect brain choline and acetylcholine pools. This observation is important since acetylcholine is an important neurotransmitter involved in cognitive function and in the neuropathology of dementia (Luque and Jaffe 2009).

Summary

The method of focused microwave radiation has been applied to the study of brain neurotransmitter metabolism in mice. This is the first study to characterize the effects of folate deficiency on numerous neurotransmitters in regional brain areas. Generally, the parent neurotransmitters (dopamine, serotonin, and NE) were not affected in young mice reared on a folate deficient diet for 3 months. However, reduced turnover of dopamine and serotonin was observed as well as decreased GABA and acetylcholine in specific brain regions of folate deficient mice. These neurochemical changes provide additional information on the way in which folate deficiency affects neuronal function and the

expression of mood disorders and cognitive function in humans. The following chapter will explore if the folate deficiency induced neurochemical changes observed in the current and previous chapter are associated with locomotor or cognitive function in mice.

CHAPTER SIX

The Effect of Folate Deficiency and Age on Behavior in Mice

Introduction

The loss of motor coordination is a common characteristic of many age-related neurological disorders along with cognitive decline (Rosenberg and Miller 1992; Thiruchelram et al. 2003; Colebrooke et al. 2006; Diaz-Hernandez et al. 2009). Moreover, epidemiological studies have linked low circulating levels of folate to neurodegeneration, and neuropsychiatric diseases, including stroke, PD, dementia, and depression (Riggs et al.1996; Joosten et al. 1997; Clake et al. 1998; McCaddon et al. 1998; Snowdon et al. 2000; Bottiglieri et al. 2000; He et al. 2004; Irizarry et al. 2005; Lamberti et al. 2005). The aged population is at a higher risk for developing folate deficiency due to reduced nutritional intake, decreased absorption, increased usage of medications that affect folate metabolism, and existing medical conditions. A recent study that highlights the role of folate in cognition showed that elderly patients supplemented with folic acid for 3 years had delayed memory loss (Durga et al. 2007). The sensitivity of cognitive function to folate status is presumed to be related to the brain's high requirement for methylation reactions (Weir and Scott 1999). However, other B vitamins such as vitamin B_{12} and B_6 that also affect the methylation pathways are also associated with cognitive decline and dementia (Werder 2010; Smith and Blumenthal 2010,; Cummings 2010).

Recent investigations have combined folate deficiency with other vitamin deficiencies in an AD mouse model (Bernardo et al. 2007) and in an apolipoprotein E

mouse model (Shea et al. 2004). A decrease in function of apolipoprotein E is associated with increased oxidative stress and is a risk factor for AD and vascular disease (Shea et al. 2004). Other investigators reported that the neurological problems of the mouse models are exacerbated in the presence of the vitamin deficiency, which was associated with cognitive impairment (Bernardo et al. 2007; Shea et al. 2004). However, these studies do not help determine the role of folate in cognitive function due to other B vitamins that were deficient in the diet. To date there are very few studies that isolate the effect of folate deficiency alone on behavior in a mouse model and no studies have been conducted in an aged model. Additionally, *tg*-MTHFR mice with a diminished capacity to recycle folate in combination with a dietary deficiency of folate have not been studied with respect to behavioral changes.

Motor coordination reflects balance, muscle strength, and patterned gait (Rustay et al. 2003). Impairments in motor performance can confound behavioral assays which evaluate learning, exploration behavior, and memory, such as TruScan and MWM. Therefore, researchers studying exploratory and cognition should also investigate coordination, whether or not motor ability is suspected to be impaired. In this study the rota rod technique will be used to assess motor coordination, TruScan to assess open field behavior, and MWM to assess learning and memory. In addition, strength will be measured by use of a grip strength meter. The present chapter aims to examine strength, coordination, open-field behavior, and cognition in aged C57BL/6J and *tg*-MTHFR mouse model exposed to low or depleted folate levels. The behavioral data was analyzed to look at the effect of age (young vs. old) and folate deficiency in young and old C57BL/6J mice. In the aged *tg*-MTHFR the behavioral data was analyzed to look at the

effect of gender (male vs. female), genotype (wild type vs. heterozygote), low folate, and the combination of diet and genotype.

Results

C57BL/6J Aged Folate Deficient Study

The time line for behavioral testing is shown in table 42. The same sets of mice were not used for repeat analysis but rather a new set of mice to avoid any affects of learning and/or memory.

Table 42. Timeline of behavioral testing in C57BL/6J mice.

Behavioral Testing Schedule												
Behavioral Parameter	Week on Diet											
	1	2	3	4	5	6	7	8	9	10	11	12
Grip Strength							√					√
Rota Rod			√			√				√		
Tru-Scan				√					√			
Morris Water Maze							√	√			√	√

Effect of age. Both 22 and 23 month old mice fed an amino acid defined control diet had less forelimb strength when compared to 6 and 7 month mice fed the same diet, respectively (6 and 22 month data not shown, Figure 27). Old mice fed an amino acid defined control diet for 3 weeks had a quicker latency to fall on the rota rod apparatus compared to young mice. This data was replicated after 6 and 10 weeks on diet. Figure 28 represents young and old mice fed an amino acid defined control diet mice after 3 and 10 weeks of treatment (data at 6 weeks not shown). There was an age-related decrease in all the open field behavior parameters measured in mice reared on an amino acid defined

control diet for 9 weeks, with the exception of stereotypic moves however; none of the differences were statistically significant (Figure 29).

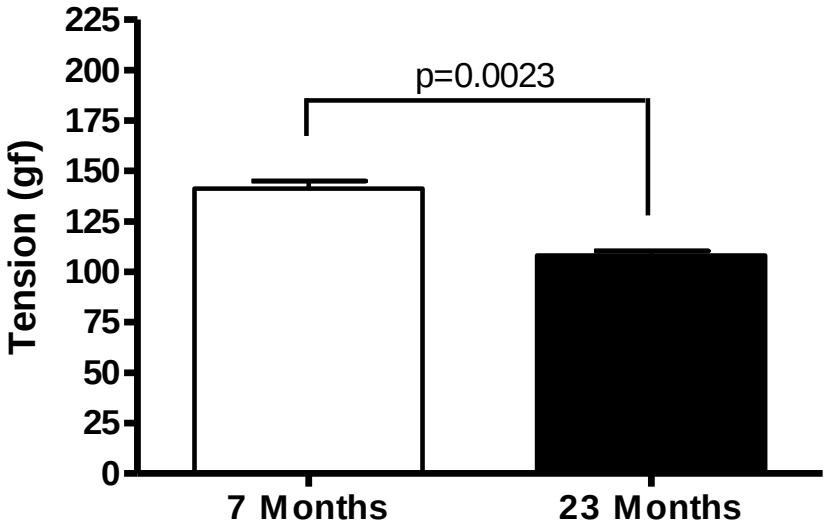

Figure 27. Effect of age on grip strength after 12 weeks on diet. Data presented as mean ± SEM (n=8;13).

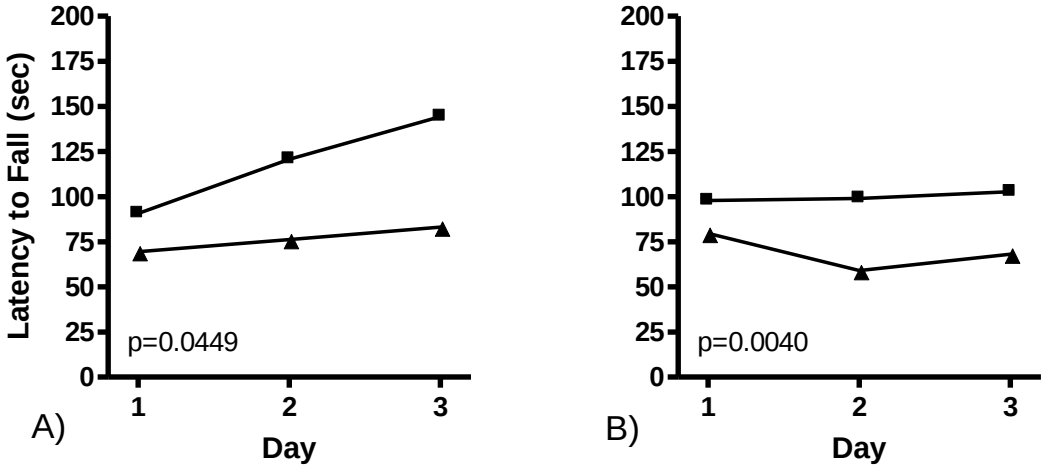

Figure 28. Effect of age on motor coordination. Data presented as mean ± SEM. A) ■=5 mo., n=14; ▲=21 mo., n=13. After 3 weeks on diet. F=4.48. B) ■=7 mo., n=10; ▲=23 mo., n=10. After 10 weeks on diet. F=10.88.

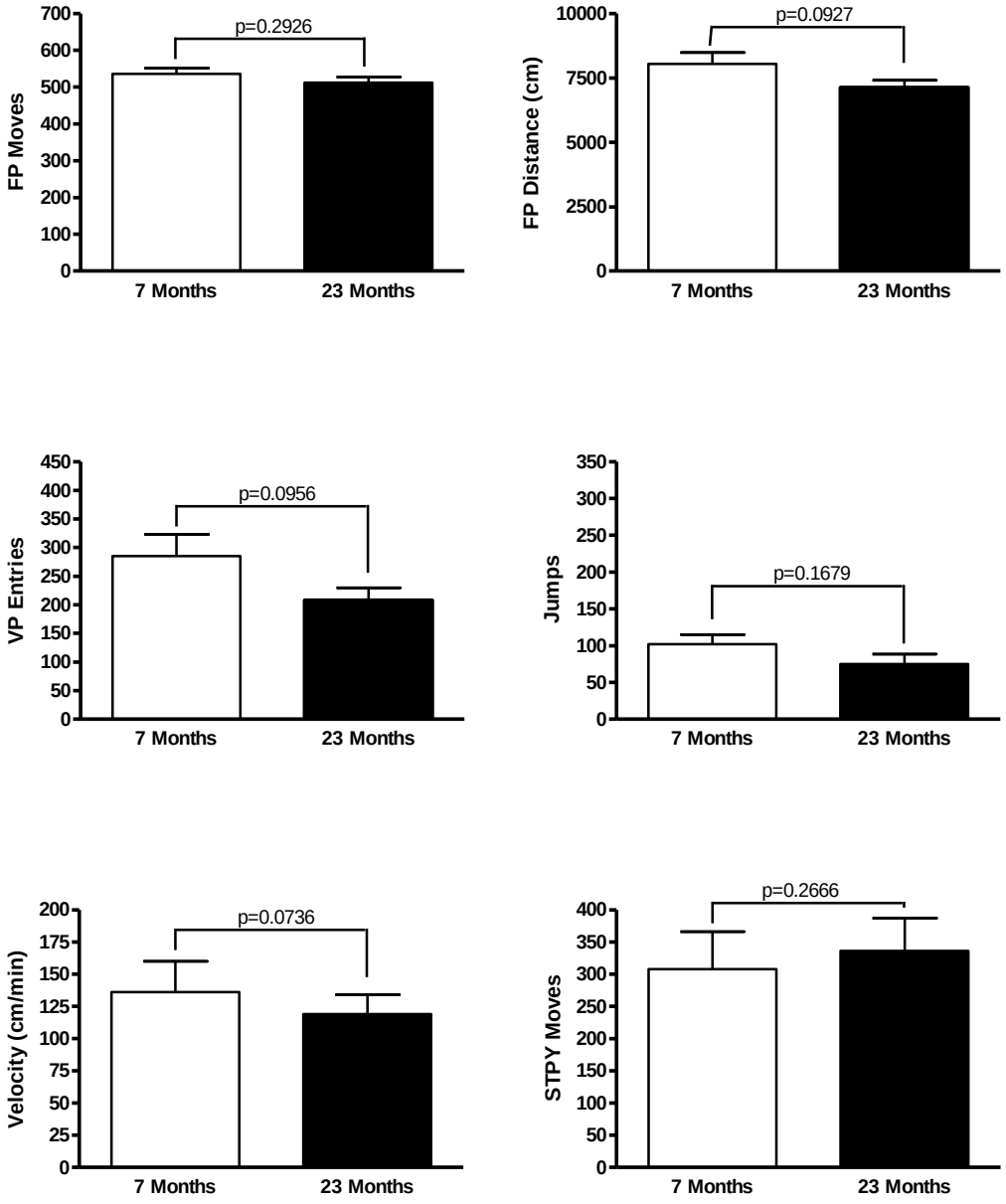

Figure 29. Effect of age on open field behavior after 9 weeks on diet. Data presented as mean ± SEM (n=10).

The effect of age on spatial memory in mice fed an amino acid defined control diet was examined after 8 and 12 weeks. After 8 weeks of treatment the 6 month old mice learned the visual portion more rapidly than 22 month old mice but on the fourth day their latencies were approximately identical (Figure 30 A). Similarly, young mice

performed better on the hidden task on days seven, eight, and nine but equaled the old mice on day ten. After 12 weeks on the diet young and old mice performed nearly identical on both visual and hidden latencies (Figure 30 B). No age-related significance in latency to platform was found after 8 or 12 weeks on control diet.

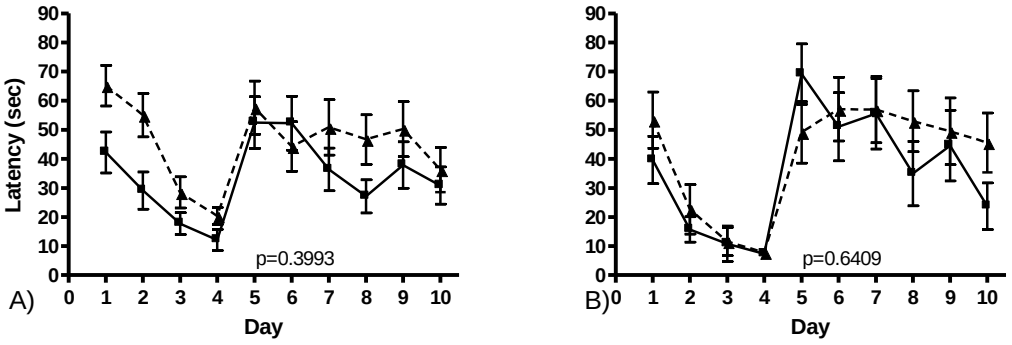

Figure 30. Effect of age on spatial memory. Data presented as mean ± SEM. A) ■=6 mo. SS=22.07, n=14; ▲=22 mo. SS 18.58, n=13. After 8 weeks on diet. F=0.72. B) ■=7 mo. SS=21.23, n=8; ▲=23 mo. SS=19.27, n=9. After 12 weeks on diet. F=0.22.

Effect of folate deficiency. There were no statistical differences in grip strength between young mice fed control diet and low folate or folate deficient diets after 7 or 12 weeks (12 week data not shown, Figure 31). However, the young mice fed a folate deficient diet for 7 weeks had reduced forelimb strength with a p value of 0.0579 compared to control diet mice. In old mice there was a significant statistical difference in grip strength induced by the folate deficient diet. Old mice reared on a folate deficient diet had an increase (nearly 50%) in neuromuscular function at both time points, 7 and 12 weeks, compared to age-matched mice fed control diet (12 week data not shown, Figure 32).

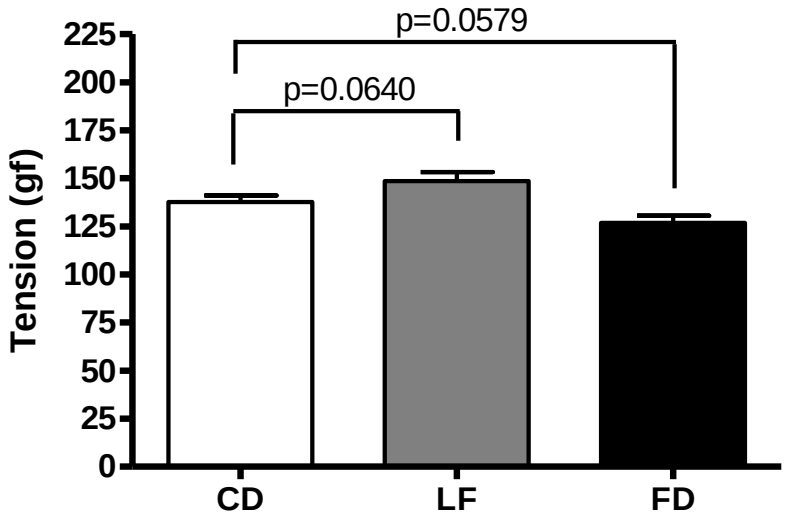

Figure 31. Effect of folate deficiency on grip strength in young mice after 7 weeks of treatment. Data presented as mean ± SEM (n=8;8;10).

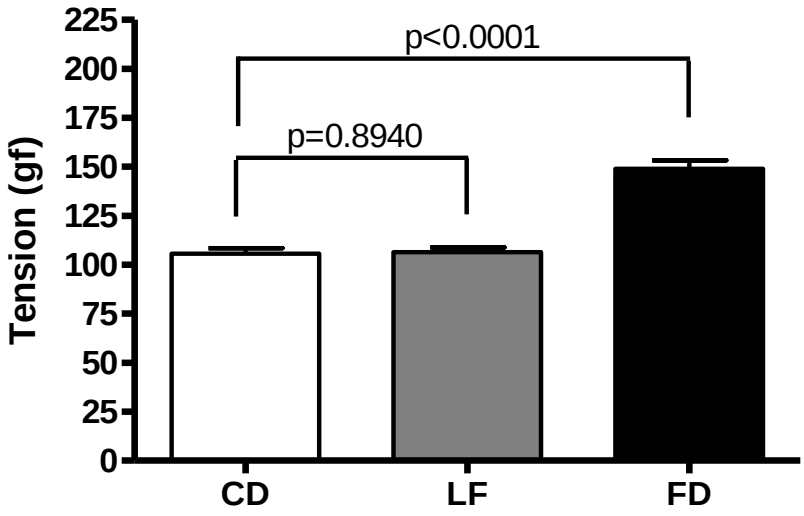

Figure 32. Effect of folate deficiency on grip strength in old mice after 7 weeks of treatment. Data presented as mean ± SEM (n=13;14;9).

The effect of folate deficiency on motor coordination was evaluated in both young and old rodents after 3, 6, and 10 weeks of study commencement (Figures 33 & 34). In young mice 3 and 6 weeks of treatment follow the same trend, with the control diet mice

having the best latency time followed by low folate and lastly, folate deficient mice. On the other hand, after 10 weeks there were no differences between the three groups in young mice. The tendency of folate deficiency lowering latency times on rota rod was not observed in old mice. Older mice that were folate deplete performed better than their control counterparts at all three test instances as did the low folate group.

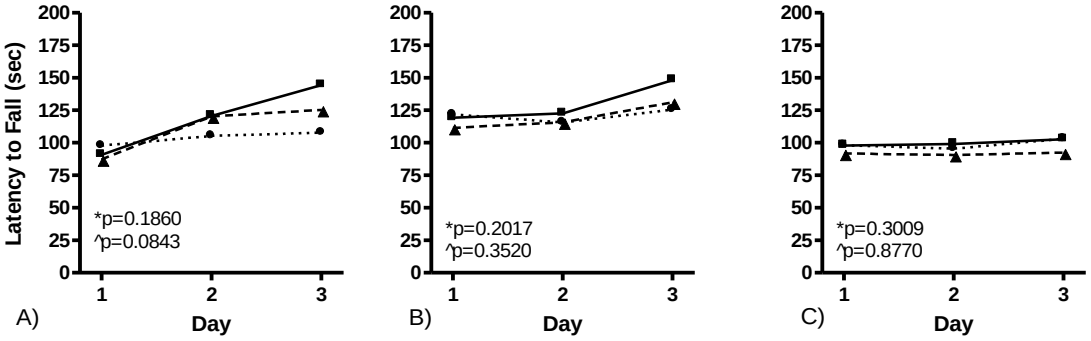

Figure 33. Effect of folate deficiency on motor coordination in young mice. Data presented as mean ± SEM ■=CD, ▲=LF, ●=FD. (n=8-14). *Compares CD to LF. ^Compares CD to FD. A) After 3 weeks on diet. *F=1.81 ^F=3.14. B) After 6 weeks on diet.*F=1.69 ^F=0.89. C) After 10 weeks on diet.*F=1.12. ^F=0.02.

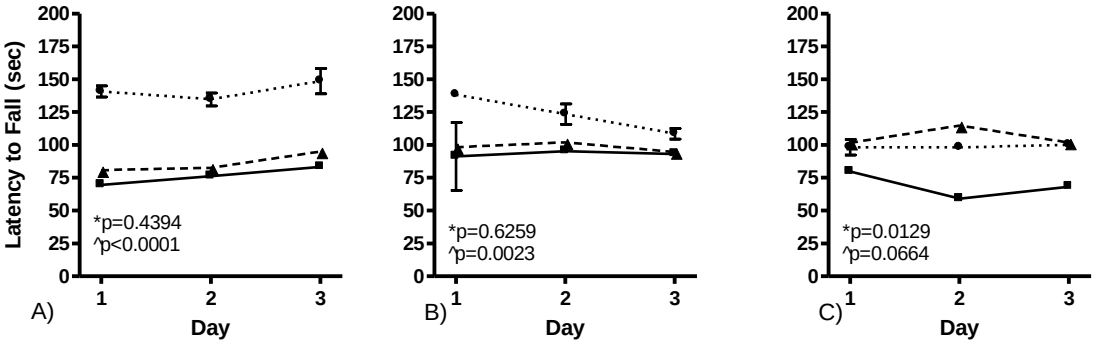

Figure 34. Effect of folate deficiency on motor coordination in old mice. Data presented as mean ± SEM ■=CD, ▲=LF, ●=FD. (n=7-14). *Compares CD to LF. ^Compares CD to FD. A) After 3 weeks on diet.*F=0.61. ^F=36.04. B) After 6 weeks on diet. *F=0.24. ^F=10.94. C) After 10 weeks on diet. *F=7.27. ^F=3.71.

Due to a malfunction in the TruScan equipment no data could be obtained for young mice at 9 weeks and for old mice at 4 weeks. After 4 weeks of experimentation young mice fed a folate deficient diet had severely decreased frontal plane moves, distance, vertical plane entries, and velocity compared to age-matched control diet mice (Figure 35). Similar results were found in old mice after 9 weeks of treatment (Figure 36). In this group folate deficiency also caused a reduction in the number of jumps in one hour. Frontal plane moves was down but the decline was not significant.

Folate deficiency did not have a significant effect on spatial memory after 8 weeks of treatment in young mice, although there was a slight effect during the hidden task (Figure 37). After 12 weeks of treatment the effect of the absence of folate is more pronounced, but it is still not statistically significant (Figure 38). The effect of folate deficiency on spatial memory was even less pronounced in old mice compared to young mice. After 8 weeks of treatment all three treatment groups averaged around the 35 seconds to reach the platform in the hidden portion of testing (Figure 39). During the twelfth week of testing all old treatment groups performed worse than after 8 weeks on the diets with no difference in swim speed between groups or trial weeks. During this round of testing control diet and folate deficient mice averaged 45 seconds and the low folate group average 38.80 seconds to latency (Figure 40).

Summary. A summary of all behavioral tests assessed (grip strength, rota rod, TruScan, and MWM) for the effect of age, and folate deficiency in young and old C57BL/6J mice is presented in table 43.

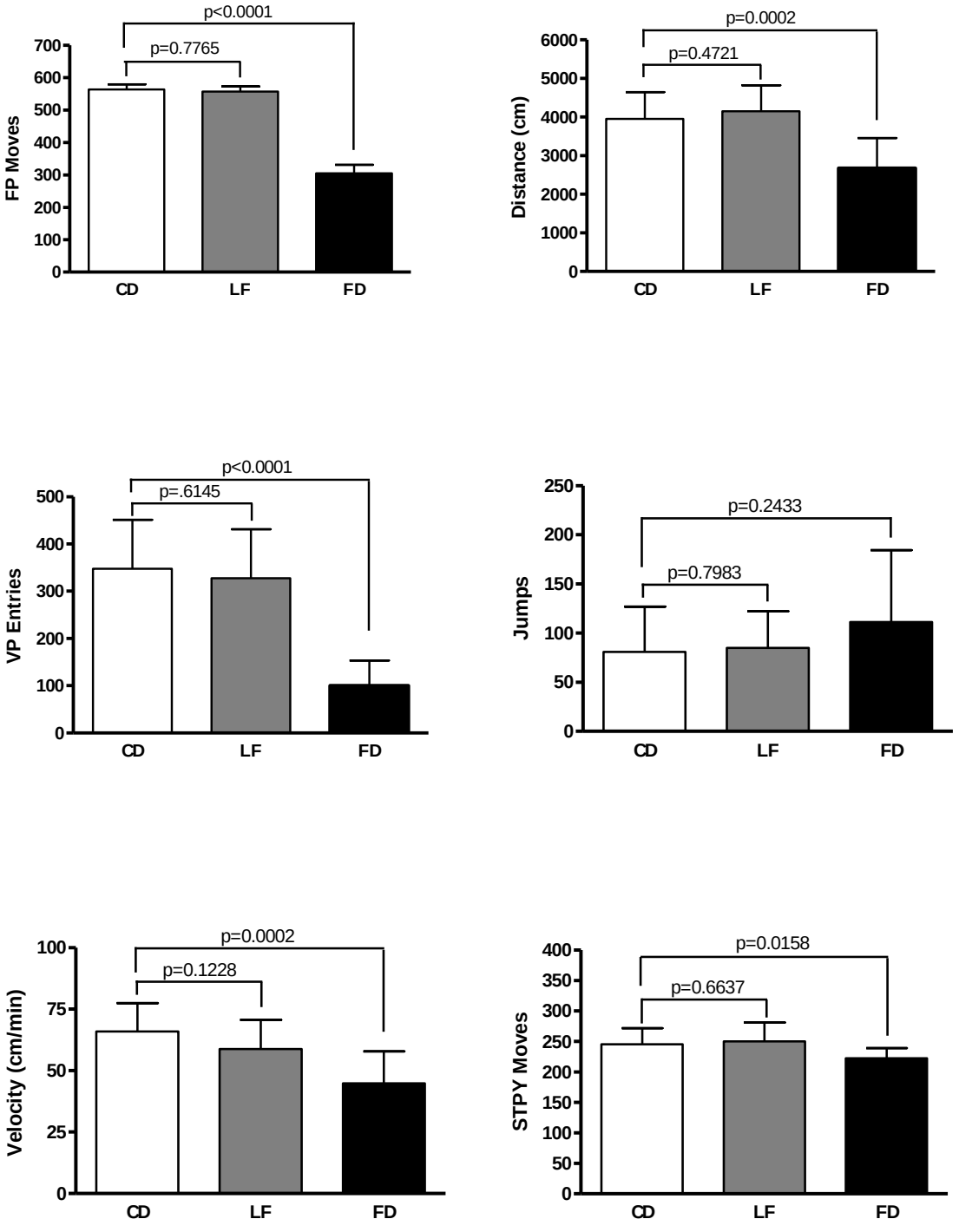

Figure 35. Effect of folate deficiency on open field behavior in young mice after 4 weeks of treatment. Data presented as mean ± SEM (n=14;13;12).

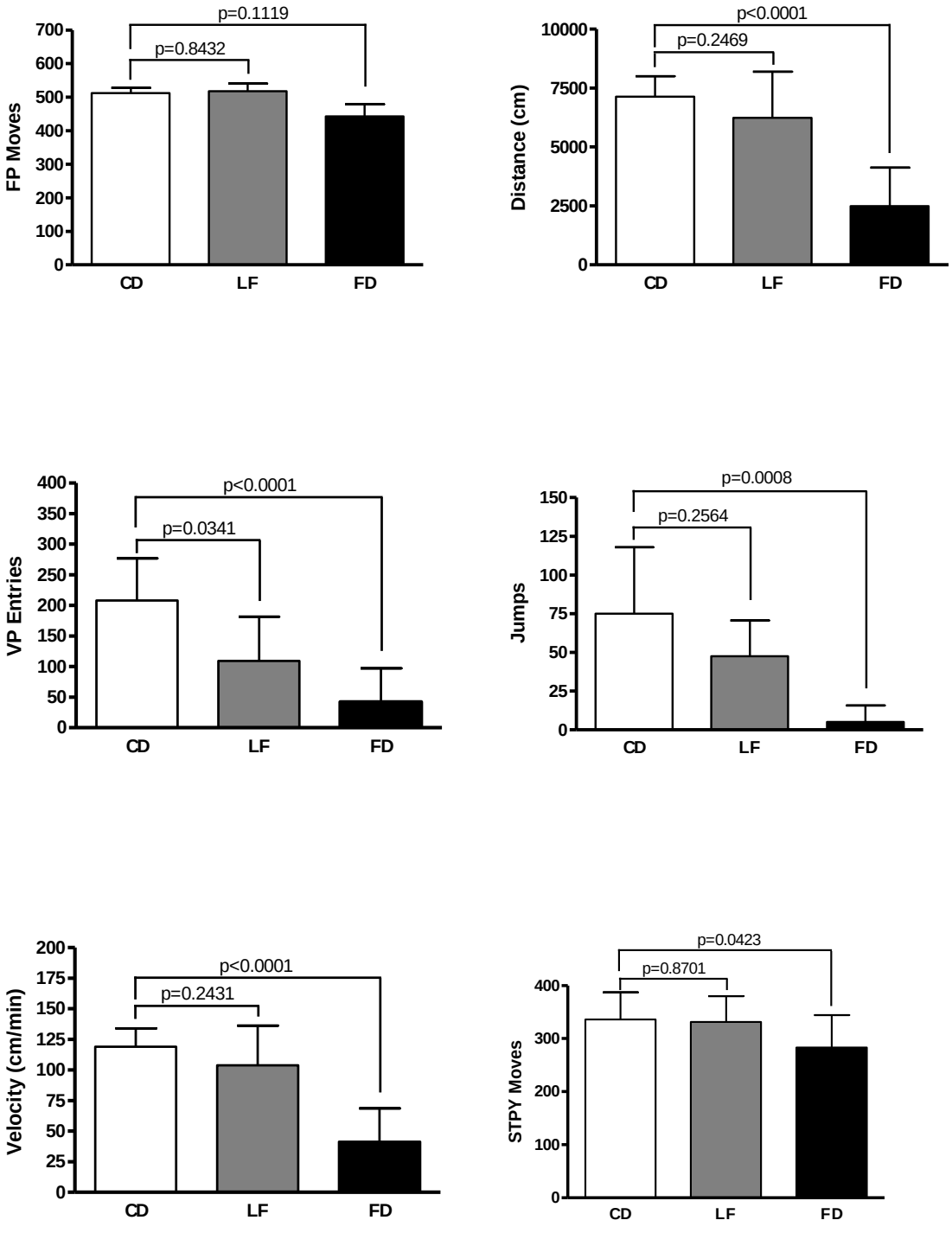

Figure 36. Effect of folate deficiency on open field behavior in old mice after 9 weeks of treatment. Data presented as mean ± SEM. (n=10;4;12).

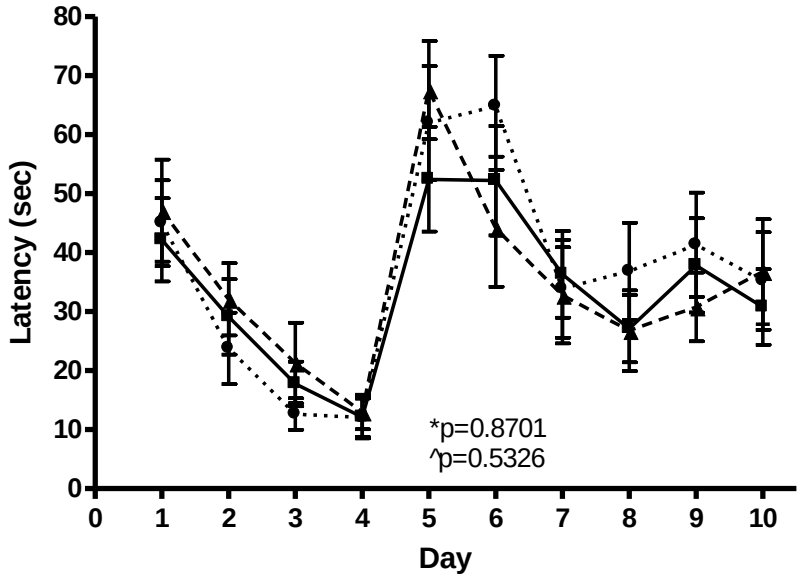

Figure 37. Effect of folate deficiency on spatial memory in young mice after 8 weeks of treatment. Data presented as mean ± SEM. ■=CD, SS= 22.07, n=14; ▲=LF, SS=21.86, n=13; ●=FD, SS=20.54, n=13. *Compared CD to LF. ^Compares CD to FD. *F=0.03. ^F=0.39.

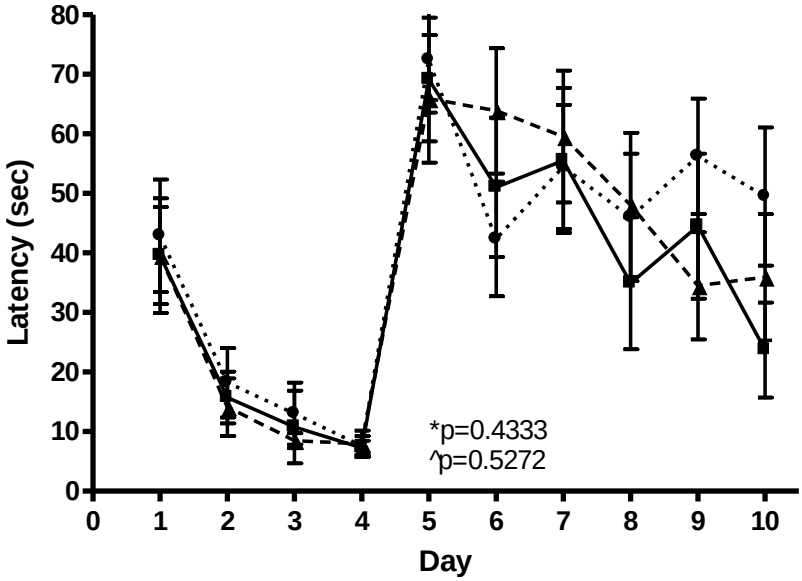

Figure 38. Effect of folate deficiency on spatial memory in young mice after 12 weeks of treatment. Data presented as mean ± SEM. ■=CD, SS=21.23, n=8; ▲=LF, SS=21.13, n=8; ●=FD, SS=18.26, n=10. *Compared CD to LF. ^Compares CD to FD. *F=0.63. ^F=0.41.

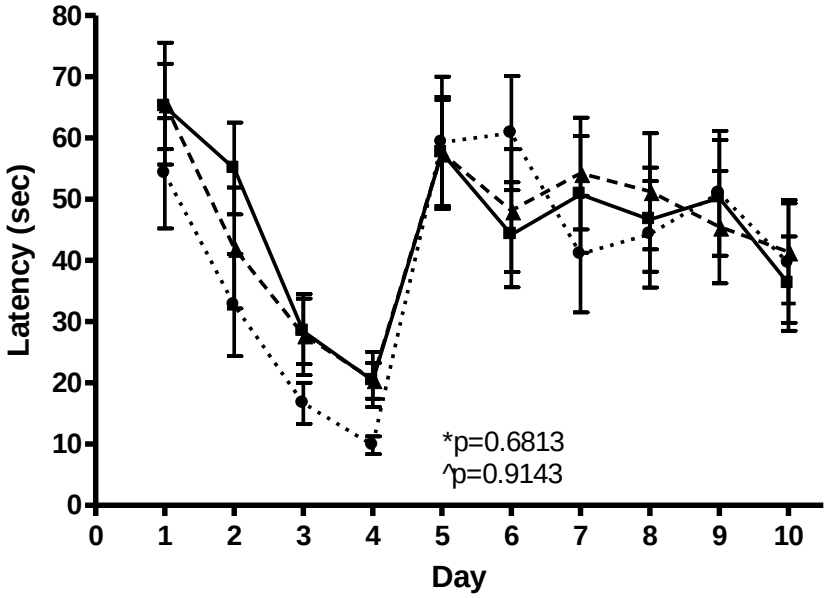

Figure 39. Effect of folate deficiency on spatial memory in old mice after 8 weeks of treatment. Data presented as mean ± SEM. ■=CD, SS=18.58, n=13; ▲=LF, SS=19.36, n=13; ●=FD, SS=19.09, n=9. *Compared CD to LF. ^Compares CD to FD. *F=0.17. ^F=0.01.

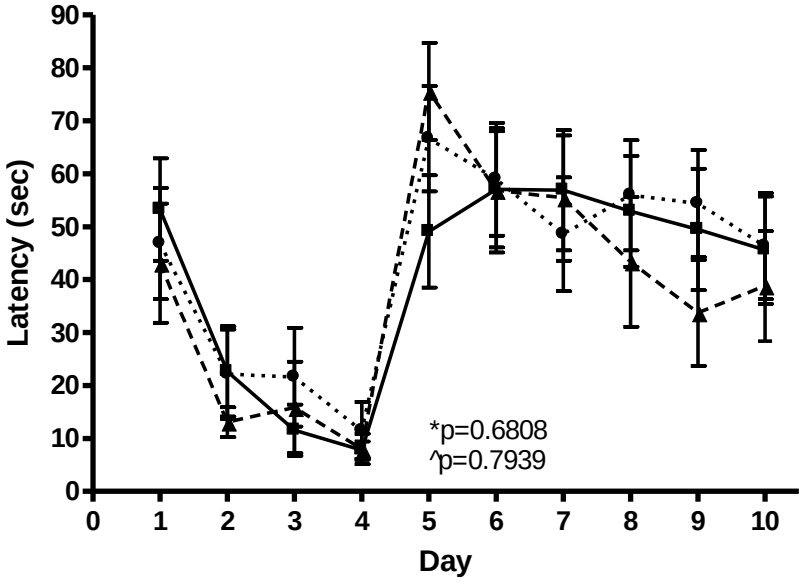

Figure 40. Effect of folate deficiency on spatial memory in old mice after 12 weeks of treatment. Data presented as mean ± SEM. ■=CD, SS=19.27, n=9; ▲=LF, SS=19.66, n=7; ●=FD, SS=18.07, n=9. *Compared CD to LF. ^Compares CD to FD. *F=0.17. ^F=0.07.

Table 43. C57BL/6J mice behavioral summary.

Test	The effect of:		
	Age	Folate in young	Folate in old
Grip Strength	Old ↓	none	FD↑
Rota Rod	Old ↓	none	FD ↑
Open Field	none	FD ↓	FD ↓
Water Maze	none	none	none

tg-MTHFR Aged Low Folate Study

The time line for behavioral testing in aged *tg*-MTHFR mice is presented in table 44. Each behavioral test was assessed once during the last month of treatment.

Table 44. Time line of behavioral testing in aged *tg*-MTHFR mice.

Behavioral Testing Schedule				
Behavioral Parameter	Week on Diet			
	21	22	23	24
Grip Strength				√
Rota Rod		√		
Tru-Scan			√	
Morris Water Maze			√	√

Effect of gender. Both wild type and heterozygous female mice registered notably less forelimb tension on the grip strength apparatus than male mice of the same corresponding genotype (Figure 41). Male wild type and heterozygous mice averaged 162 gf whereas; the wild type and heterozygous females averaged 145 gf.

On day one of testing females appear to have better locomotor capability than males. However, as testing continued the sexes nearly equivocate on day three. Female *tg*-MTHFR aged mice have slightly higher motor ability than male *tg*-MTHFR aged mice in both genotypes but the data is not statistically significant once repeated measures and weight are taken into consideration (Figure 42)

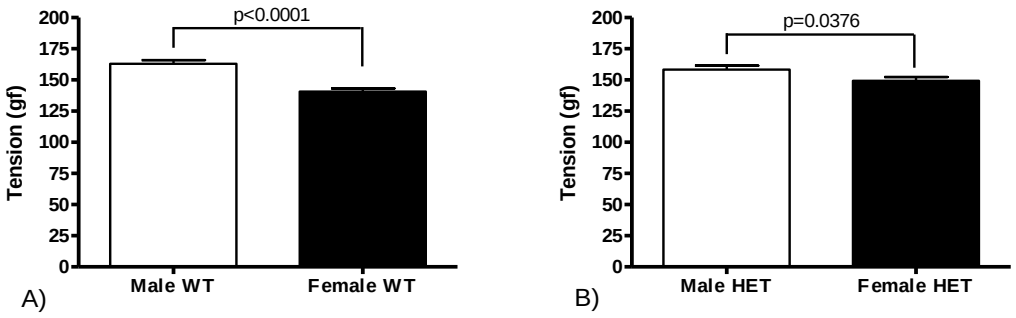

Figure 41. Effect of gender on grip strength in *tg*-MTHFR mice. Data presented as mean ± SEM. A) Wild type mice (n=10;9). B) Heterozygous mice (n=8;10).

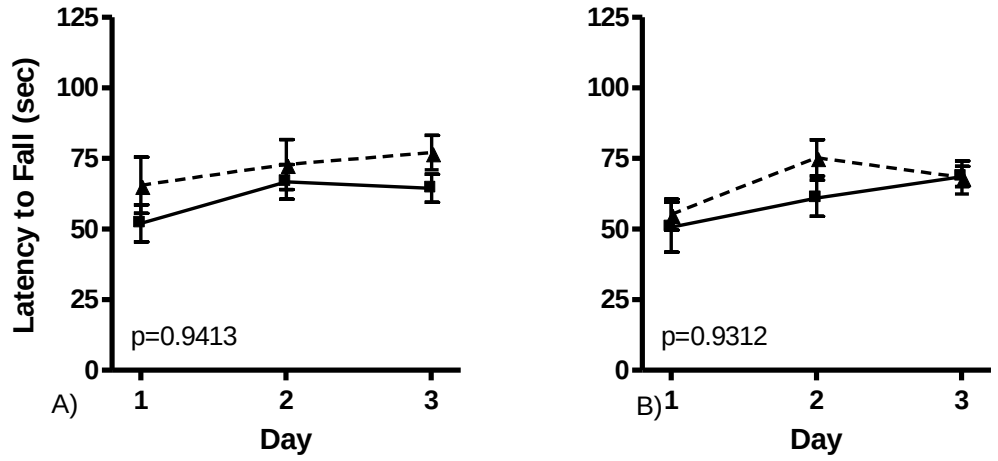

Figure 42. Effect of gender on motor coordination in tg-MTHFR mice. Data presented as mean ± SEM. ■=male,▲=female A) Wild type mice (n=10;9) F=0.01. B) Heterozygous Mice (n=8;10) F=0.01.

Female mice of both genotypes showed an increase in frontal plane distance and velocity compared to their matching male counterparts (Figure 43). A decrease in stereotypic moves was shown in female wild type mice compared to male wild type mice but this was not repeated in the heterozygous mice to a significant degree. Although not significant female mice also had an increase in jumps and vertical plane entries in both genotypes evaluated against male mice.

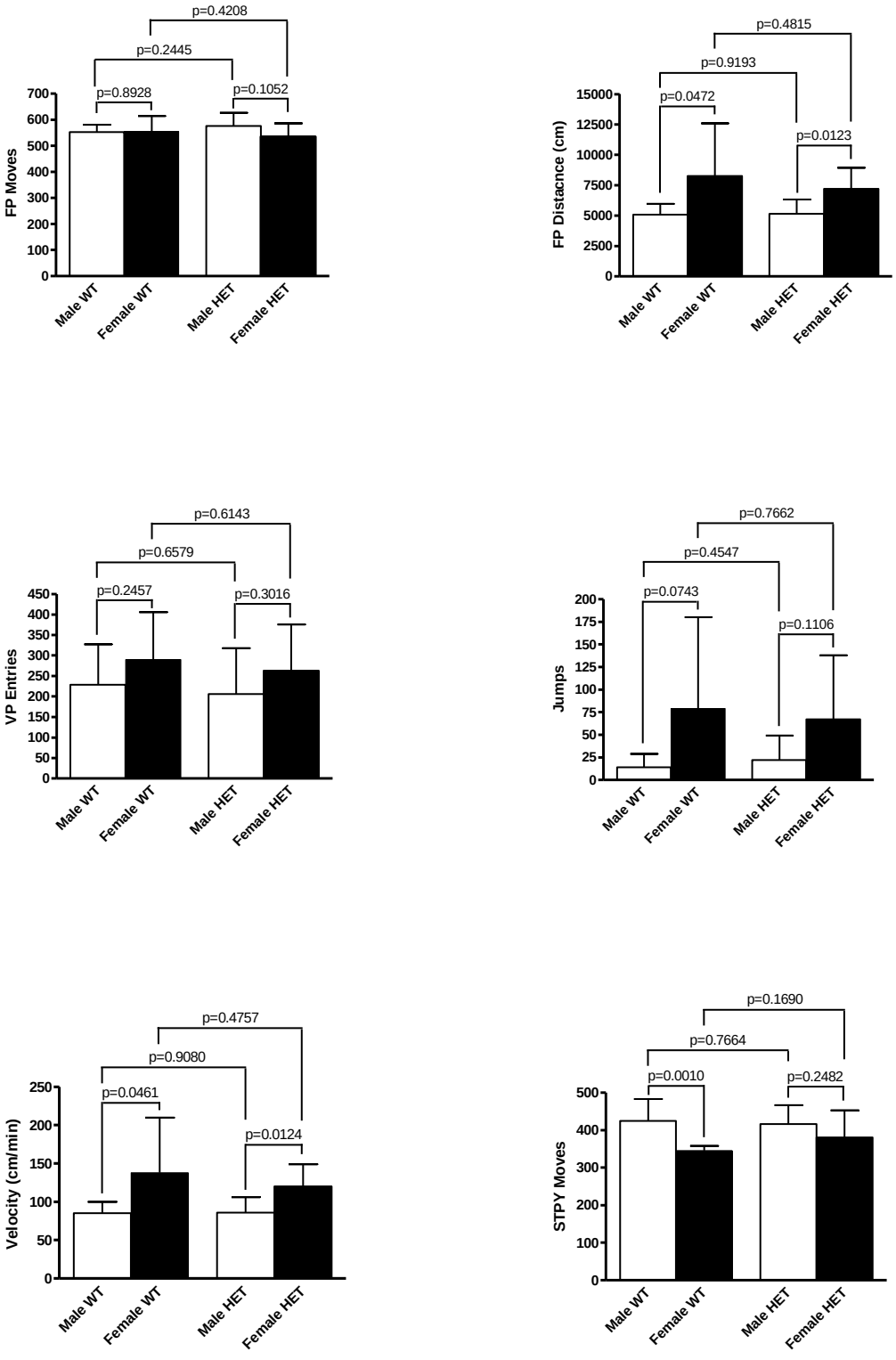

Figure 43. Effect of gender and genotype on open field behavior in *tg*-M*THFR* mice. Data presented as mean ± SEM (n=9;9;8;10).

During the final 2 weeks of treatment the mice were tested for spatial memory using the MWM. Wild type and heterozygous male and female mice performed in a similar way in this task during both the visual and hidden days of testing (Figure 44).

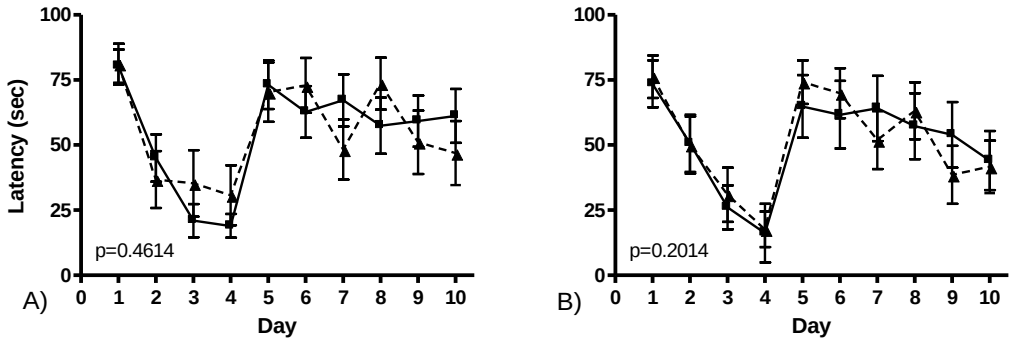

Figure 44. Effect of gender on spatial memory in *tg*-M*THFR* mice. Data presented as mean ± SEM. ■=male, ▲=female A) Wild type mice (n=10;8) F=0.56. B) Heterozygous Mice (n=8;9) F=1.71.

Effect of genotype. The effect of genotype on grip strength was assessed in both males and females during the final month of treatment. There were no differences in grip strength due to genotype in either male or female mice. However, female heterozygous mice had slightly greater grip ability than wild type mice but it was not statistically different (Figure 45). On the rota rod task all mice fed a control diet remained on the rod for about 75 seconds on the third day of testing, thus there were no significant differences in motor coordination due to genotype (Figure 46). Additionally, genotype did not impact open field behavior (Figure 43) or spatial memory (Figure 47) in aged male or female *tg*-MTHFR mice. However, latency to reach the visual platform on the final day of cue testing was under 25 seconds and 50 seconds for hidden testing for all groups fed a control diet.

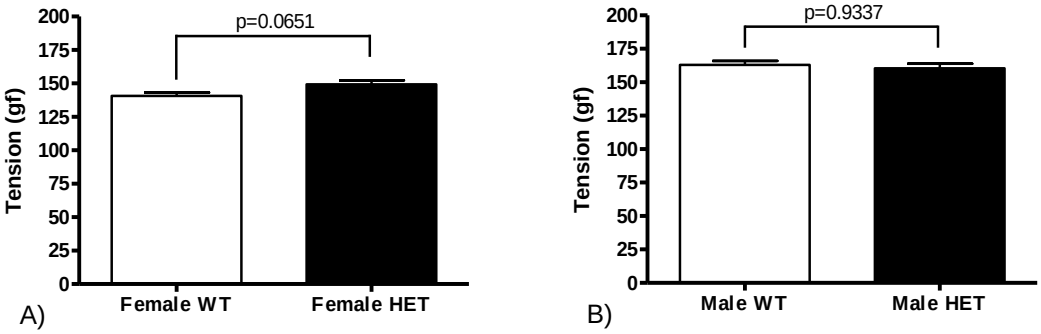

Figure 45. Effect of genotype on grip strength in *tg*-MTHFR mice. Data presented as mean ± SEM. A) Female mice (n=9;10). B) Male mice (n=10;8).

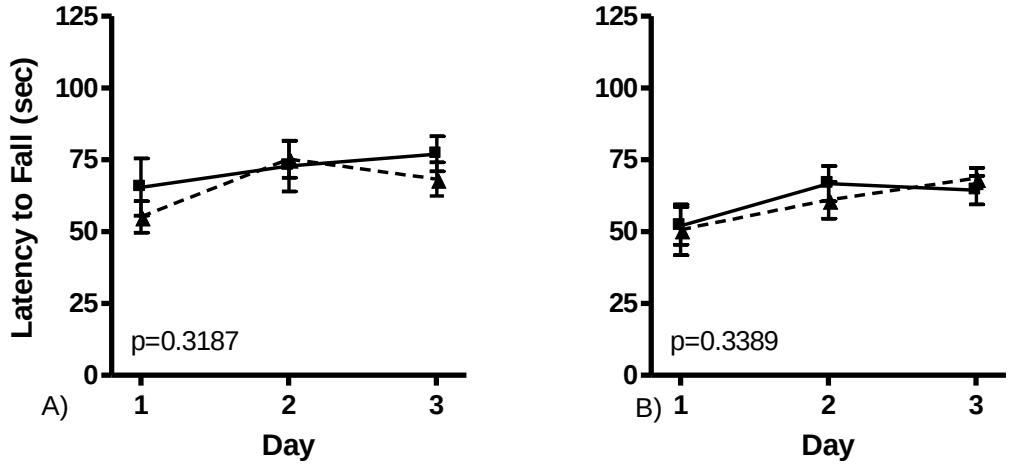

Figure 46. Effect of genotype on motor coordination in *tg*-MTHFR mice. Data presented as mean ± SEM. ■=WT,▲=HET A) Female mice (n=9;10) F=1.09. B) Male mice (n=10;8) F=1.02.

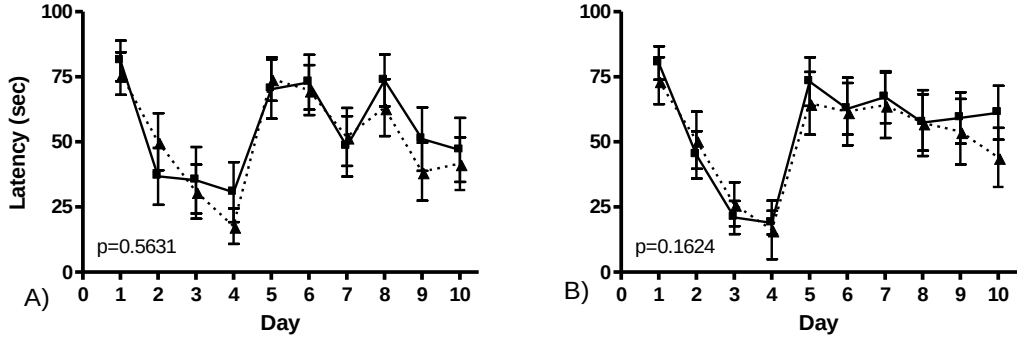

Figure 47. Effect of genotype on spatial memory in *tg*-M*THFR* mice. Data presented as mean ± SEM. ■=WT,▲=HET A) Female mice (n=8;9) F=0.34 B) Male mice (n=10;8) F=2.08

Effect of low folate. Male and female mice fed a low folate diet exerted less forelimb strength compared to mice fed a control diet of matching gender and genotype (Figure 48); the divergence was greatest in males. There was also a considerable difference between wild type mice fed a control diet and heterozygous mice fed a low folate diet in both genders. However, it did not seem that low folate in combination with a heterozygous genotype compounded the effect on grip strength in either gender.

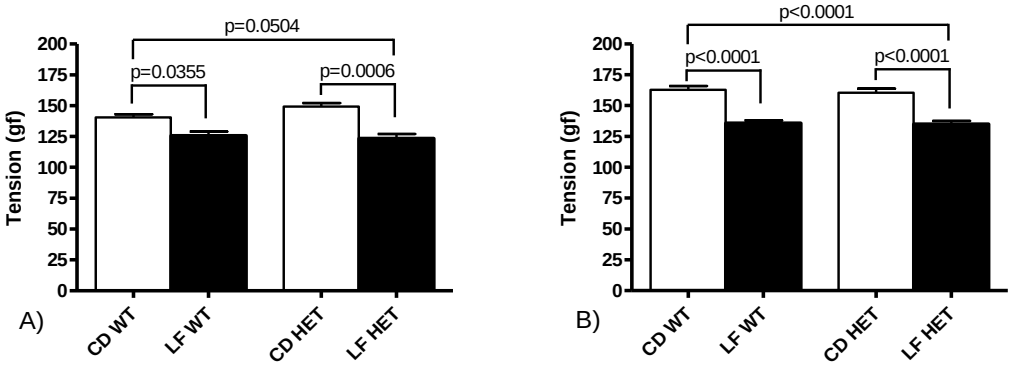

Figure 48. Effect of low folate on grip strength in *tg*-MTHFR mice. Data presented as mean ± SEM. A) Females (n=9;9;10;9). B) Males (n=10;10;8;10).

There were no statistical differences caused by a low folate diet on latency to fall by assessment of the rota rod apparatus in female or male mice. Figures 49A and 50A contain all four groups across 3 days of testing. Figures 49B-C and 50B-C depicts the latency differences between low folate and control mice of different genotypes. Figures 49D and 50D represent the effect of the combination of low folate and heterozygosity compared to control diet wild type mice.

None of the six parameters assessed in the open field behavior test were significantly affected by treatment with a low folate diet in female and male mice (Figures 51 and 52). There was an insignificant decline recorded in distance, velocity,

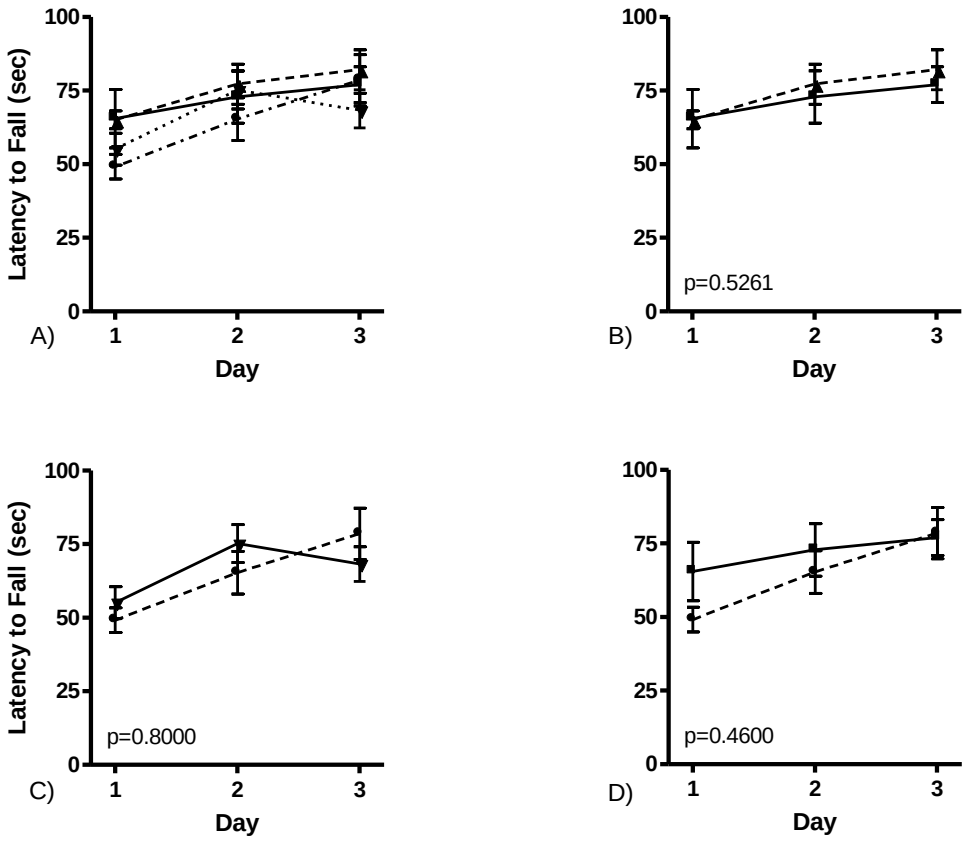

Figure 49. Effect of low folate on motor coordination in female *tg*-MTHFR mice. Data presented as mean ± SEM. ■=CD WT,▲=LF WT, ▼=CD HET, ●=LF HET (n=9;8;10;8). A) All four groups B) Wild type: CD vs. LF. F=0.41. C) Heterozygous: CD vs. LF. F=0.06. D) CD WT vs. LF HET. F=0.54.

vertical plane entries, and jumps in mice fed a low folate diet for 6 months. Statistical analysis only showed a significant difference between wild type mice fed a control diet and heterozygous mice fed a low folate diet in vertical plane entries for female mice. Thus, in female mice low folate status alone does not have an effect on open field behavior but it does in combination with genotype.

Decreased folate status alone or in combination with a heterozygous genotype did not have any influence on latency to reach the hidden platform during MWM (Figures 53 and 54).

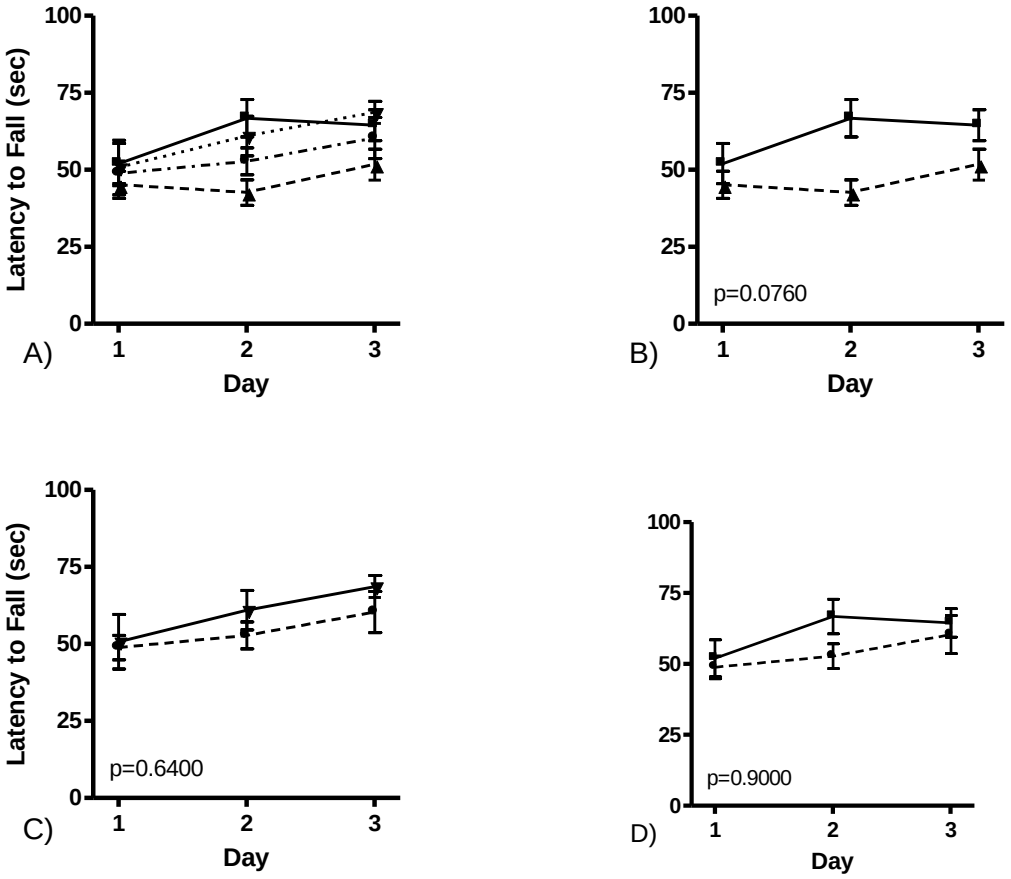

Figure 50. Effect of low folate on motor coordination in male *tg*-MTHFR mice. Data presented as mean ± SEM. ■=CD WT,▲=LF WT, ▼=CD HET, ●=LF HET (n=10;10;8;10). A) All four groups B) Wild type: CD vs. LF. F=3.33. C) Heterozygous: CD vs. LF. F=0.64. D) CD WT vs. LF HET. F=0.02.

Summary. A summary of all behavioral tests assessed (grip strength, rota rod, TruScan, and MWM) for the effect of gender, genotype, and low folate in aged *tg*-MTHFR mice is presented in table 45. Generally, low folate only played a role in grip strength. Likewise grip strength was also affected by gender. Additionally, open field behavior was affected by gender. Genotype did not affect any of the behavioral parameters assessed.

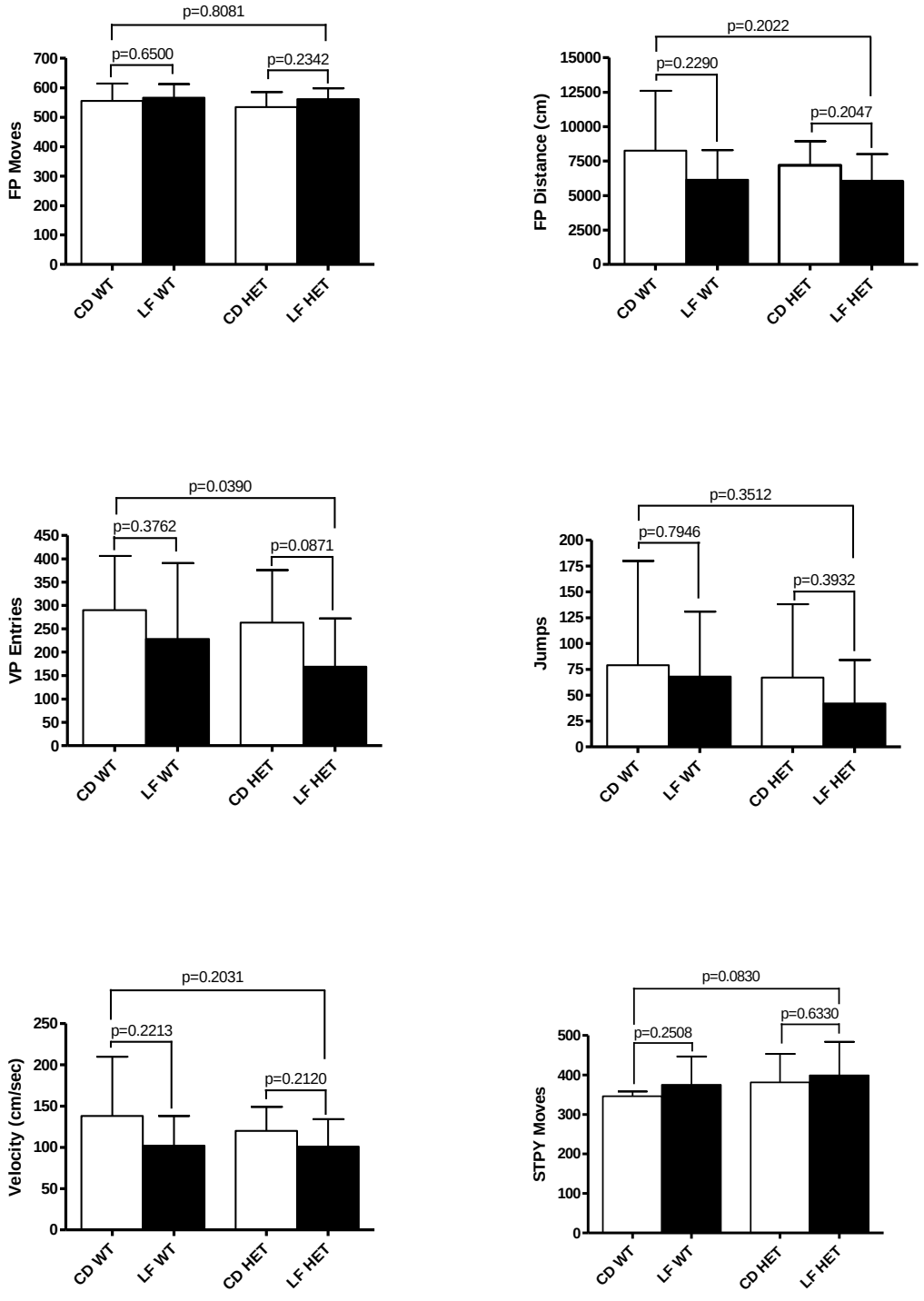

Figure 51. Effect of low folate on open field behavior in female *tg*-MTHFR mice. Data presented as mean ± SEM (n=9;8;10;8).

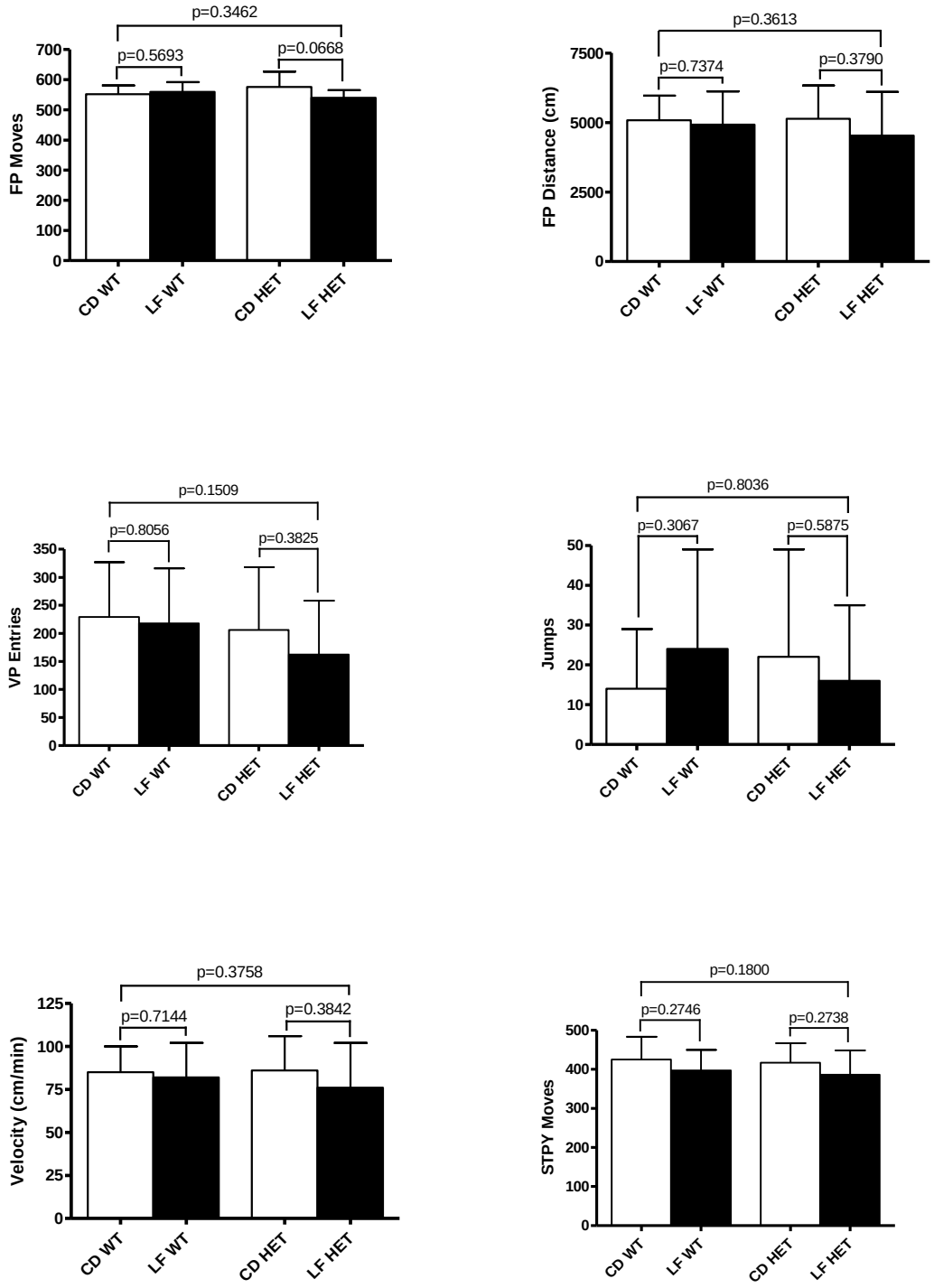

Figure 52. Effect of low folate on open field behavior in male *tg*-MTHFR mice. Data presented as mean ± SEM (n=9;11;8;10).

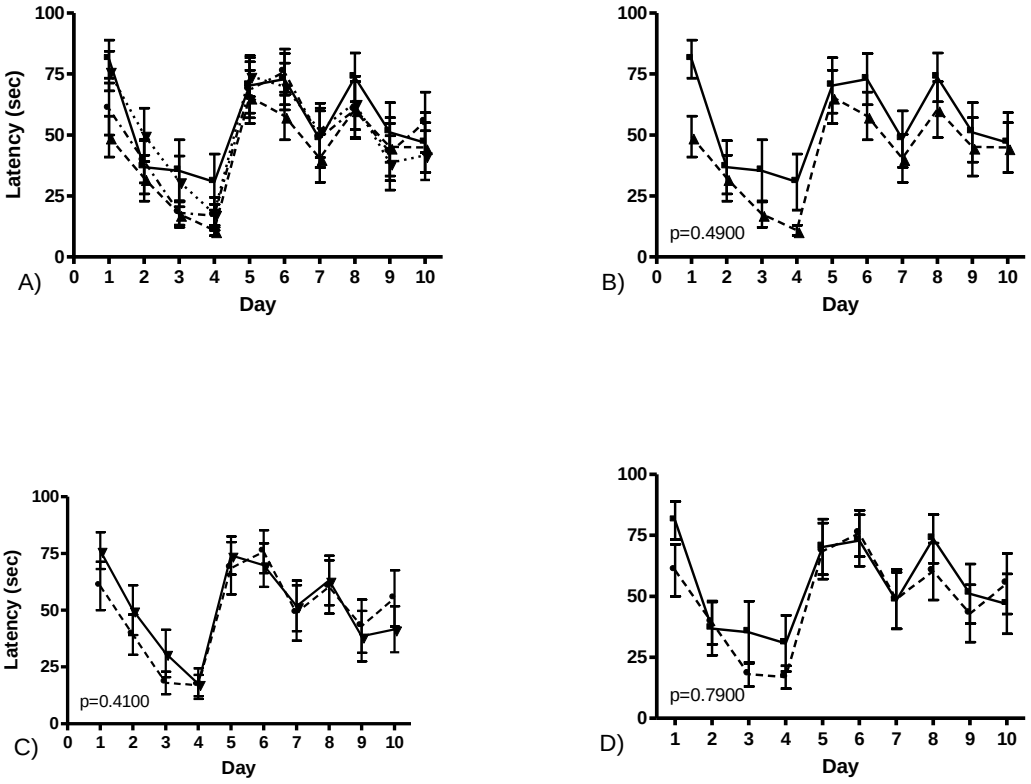

Figure 53. Effect of low folate on spatial memory in female *tg*-MTHFR mice. Data presented as mean ± SEM. ■=CD WT,▲=LF WT, ▼=CD HET, ●=LF HET (n=8;8;9;8). A) All four groups B) Wild type CD vs. LF. F=0.48. C) Heterozygous CD vs. LF. F=.69. D) CD WT vs. LF HET. F=0.07.

Discussion

C57BL/6J Aged Folate Deficient Study

Effect of age. It is well established that grip strength decreases with an increase in age in humans (Kjerland 1953; Anderson and Cowan 1996; Schmidt and Toews 1970; Kellor et al. 1971). Recently, grip strength testing has gained increasing recognition as a useful clinical marker of sarcopaenia (loss of muscle mass and strength with age) and also as a general measure of fragility and aging (Morley et al. 2001; Syddall et al. 2003;

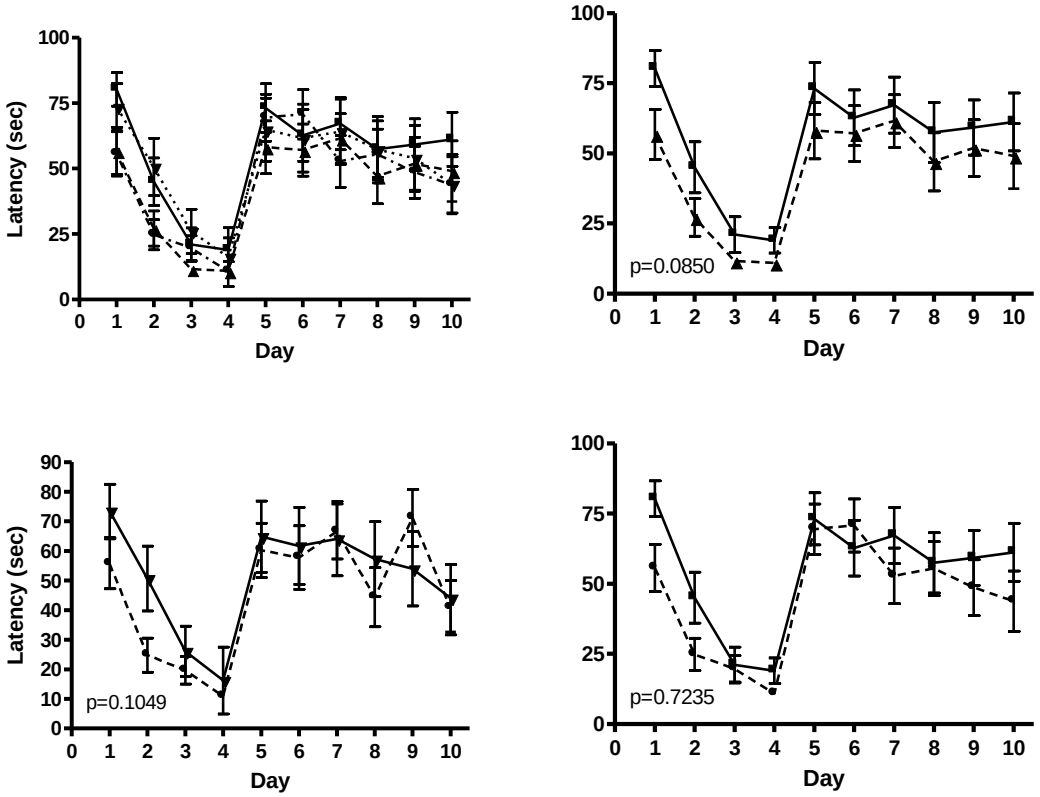

Figure 54. Effect of low folate on spatial memory in male *tg*-MTHFR mice. Data presented as mean ± SEM. ■=CD WT,▲=LF WT, ▼=CD HET, ●=LF HET (n=10;8;10;10). A) All four groups B) Wild type CD vs. LF. F=3.07. C) Heterozygous CD vs. LF. F=2.71. D) CD WT vs. LF HET. F=0.13.

Table 45. Aged *tg*-MTHFR mice behavioral summary.

Test	The effect of:			
	Gender	Genotype	Folate in female	Folate in male
Grip Strength	Female ↓	none	LF ↓	LF ↓
Rota Rod	none	none	none	none
Open Field	Female ↑↓	none	LF HET ↓	none
Water Maze	none	none	none	none

Sayer et al. 2006). The results presented in this chapter show that age has a significant detrimental effect on forelimb grip strength in C57BL/6J mice reared on an amino acid defined control diet (Figure 27). This observation is sustained by earlier studies. Ingram

performed three grip strength trials on mice ranging from 4 to 34 months of age and found that strength ability decreases with an increase in chronological age (Ingram et al. 1983). A similar test that measures the ability of a mouse to grasp a horizontal bar and remain suspended for an allotted amount of time also established that mice at 31 months of age perform inferior to 3 month old mice (Dean et al. 1981). A difference in weight could potentially affect grip strength outcome, however, statistical analysis of grip strength was corrected for weight. Thus, the decrease in grip strength in aged mice is not attributed to differences in weight. These reports along with this study indicate there is a significant age related decline in grip strength in C57BL/6J mice possibly due to sarcopaenia.

It was shown that old mice fed an amino acid defined control diet performed poorly on the rota rod task compared to young mice fed the same diet (Figure 28). This data is supported by previous studies that have shown a decrease in latency to fall with an increase in age in various mouse models (Dean et al. 1981; Ingram et al. 1983; Serradj and Jamon 2007). The consensus is that once the decrease in locomotor activity has been established in late adulthood it does not become markedly worse in old age (Gower and Lamberty 1993; Dean et al. 1981; Serradj and Jamon 2007).

Age did not impact open field behavior in mice fed an amino acid defined control diet (Figure 29). For the most part researchers have concluded that the exploratory activity decreases with an increase in age (Dean et al. 1981; Gower and Lamberty 1993; Ingram et al. 1981). However, these earlier studies used very different protocols than the one used in this study. For instance measurements were very subjective due to analysis by an observer, and the only parameter established was mean activity.

Deterioration of learning and memory is a common characteristic of the aging process in humans and rodents (Frick 1999). Many studies in mice and rats have found that there is considerable difference in MWM performance between young and old mice, whereas age is directly proportional to latency to reach the hidden platform (Brandeis et al. 1989; Fordyce and Wehner 1993; Bellush et al. 1996; Wong and Brown 2007). However, Frick published data concluding that there were not any significant age-related declines in spatial memory tested by MWM when he compared 5, 17, and 26 month old C57BL/6J mice (Frick 1999). In this study it appears that aged mice acquired the spatial task more slowly but over the testing protocol they improve nearly to the level of the young mice. It is likely that a more difficult protocol (less days of learning) may have shown significant differences between young and old mice (Figure 30). However, during preliminary testing of the MWM protocol 3 month old mice were tested and reached latency to find the platform in less than 10 seconds on the final day of testing. Data in the literature also indicates that non-impaired mice have the ability to find the hidden platform in less than 15 seconds (Frick 1999; Troen et al. 2008; Wong and Brown 2007). Thus, it appears that the young mice (6-7 months) used in this study may have some degree of spatial memory impairment since latency to reach the platform was well above 15 seconds (mean = 27.6 seconds).

It is important to note that individuals of similar advanced age often present with varying degrees of impairment, whether it be memory or locomotor ability. Generally, in a large group of aged subjects some will exhibit no memory or locomotor deficits whereas others may have marked dysfunction (Collier and Coleman 1991). For instance, in humans spatial memory has been shown to decrease as little as 30% and as much as

80% with age (Moore et al. 1984; Evans et al. 1984; Moffat et al. 2001, Jansen 2010). In this study although the data from each age group of mice may appear uniform, there were great differences between mice in the same age group, except data generated from the grip strength task. This point is important because most likely mice like humans have varying degrees of age-related loss of behavioral functions.

Effect of folate deficiency. It was hypothesized that folate deficiency may adversely affect muscle mass and strength, and that it may be exacerbated in aged mice. However, folate deficiency played no role in grip strength ability in young mice but did have a propitious effect in old folate deficient mice compared to age-matched mice (Figures 31 and 32). Folate is required for the synthesis of methionine which is incorporated into proteins, and also for the synthesis of purines and pyrimidines required for DNA synthesis. Because muscle mass represents the balance between muscle cell replication and protein synthesis and muscle protein breakdown and cell death it is unclear why old mice fed a folate deficient diet had an increase in grip strength. To date this is the first study that investigated the role of folate in grip strength in both young and old mice.

Old folate depleted C57BL/6J mice showed an increase in latency to fall on the rotating rod with respect to control diet mice of the same age, however, this finding was not evident in the young mouse model (Figures 33 and 34). Previous studies have also found that young rats and mice reared on a folate deplete diet for at least 10 weeks perform analogous to the control diet group (Vitali and Clark 2004; Troen et al. 2008). One of the downfalls of the rota rod apparatus is that some mice have the ability to grasp the rod and make several complete rotations without falling, thus strength plays a factor

during the rotations rather than motor coordination. In the old folate deficient mice it is postulated that a decrease in size and an increase in strength could account for increased rota rod ability.

Open field behavior was significantly decreased in both young and old folate deficient mice (Figures 35 and 36). Generally, the differences were declines in distance, velocity, stereotypic moves, and total distance traveled. Since folate deficiency did not cause any negative effects on locomotor ability, as previously determined by the rota rod test, these findings suggest that folate status may affect the emotional aspects of open filed behavior, such as anxiety. It would be of interest to measure anxiety levels in these mice either by elevated Plus Maze, or a light-dark exploration test. These tests were not available when the experiments with folate deficient mice were performed. The elevated Plus Maze has been successfully used as a test for anxiety-like behavior in mice. The mouse is given the option of exploring protected (enclosed) or unprotected (open) arms that are elevated 1 meter above the ground. The degree of anxiety is assessed by measuring time spent in the open arms. However, in 2008 Lalonde and colleagues reported that young vitamin B deficient Blab/c mice are more active in open field behavior and in the protected arms of the elevated Plus Maze, activity in the unprotected arms was not discussed (Lalonde et al. 2008).

No statistically significant effect of folate deficiency on water maze performance was demonstrated in young and old mice (Figures 37-40). However, as previously mentioned the control mice used in this study seem to have some degree of impairment on the MWM task, thus the effects of folate deficiency may be masked by the impairment in the control diet mice. Troen and colleagues found folate-related deterioration in spatial

memory in an APP over-expressing mouse model (Bernardo et al. 2007). More recently, he reported that young Sprague Dawley rats fed a folate deficient diet for 10 weeks have an escape latency of 24.2 seconds and the control subject 16.6 seconds, which is statistical significantly (Troen et al. 2008). However, in the same year this group published a paper stating the C57BL/6J mice fed treatment diet lacking folate, vitamin B_{12} and B_6 for 10 weeks were not significantly affected on spatial memory compared to control mice. Although the deficient mice did have deficits in the probe and reversal task, which are much harder memory tests (Troen et al. 2008). Similarly, other investigators found no cognitive impairments assessed by MWM in 3 week old TgCRND8 mice, a mouse model of AD, fed a diet low in folate, vitamin B_{12} and vitamin B_6 for 2 months (Fuso et al. 2008). Folic acid supplementation in 20 month old rats did not alter MWM performance compared to placebo mice (Lalonde et al.1992). Furthermore, treated and untreated 20 month old rats were able to find the hidden platform in less than 10 seconds on the third day of hidden testing (Lalonde et al. 1992). These conflicting results may be attributed to difference in genetic background of the rodents evaluated and protocol variations such as: number and length of trials, exact age of rodents, starting locations, and diameter of the pool used in the study. More importantly many of the previously mentioned studies do not employ a diet solely deficient in folate but instead their diets lack folate and several other important nutrients such as vitamin B_{12} and B_6, thereby resulting in a more folate deficient status and consequently a more severe HHcy mouse model compared to the folate deficient mouse model studied in this dissertation.

tg-MTHFR Aged Low Folate Study

Effect of gender. Female *tg*-MTHFR mice have decreased forelimb grip strength ability than male mice (Figure 41). Similarly, human studies have also reported more powerful grip strength in men compared to women (Kjerlan 1953; Anderson and Cowan 1966; Kellor et al. 1971; Kamarul et al. 2006; Alfaro-Acha et al. 2006). The gender difference in grip strength is presumably due to differences in muscle mass between males and females. Female *tg*-MTHFR mice had increased velocity, distance and deceased stereotypic moves compared to male mice in both genotypes (Figure 43). This finding is supported by a study that compared male and female 90 day old Swiss albino mice in an open field setting for 5 minutes and found that the female mice were much more active than male mice; this publication did not specify the parameters increased (Palanza 2001). Earlier studies have also reported higher ambulation in female rats compared to males during many different stages of life (Seliger 1977; Masur et al. 1980; Joseph and Gallagher 1980). The gender difference in exploratory behavior is not attributed to a deficit in motor coordination evident by the rota rod task; no differences in gender differences were present (Figure 42). Likewise, gender did not affect learning and spatial memory explored by the MWM test in this study (Figure 44). Other research groups have also observed no differences between males and females on spatial memory in either triple transgenic AD mice at 18 months of age or in Evan rats at 6 months of age supporting this experimental finding (Bucci et al. 1995; Gulinello et al. 2009).

Effect of genotype. There were no differences in any of the behavioral parameters measured between the MTHFR wild type and heterozygous mice fed a control diet

(Figures 43 and 45-47). This is consistent with the fact that humans with the C677T genotype normally do not have impaired sensorimotor or cognitive function (Durga et al. 2006). Furthermore, a study of an aged population containing various MTHFR polymorphisms found that those having the C677T genotype do not appear to be related to fragility, suggesting that folate dependent one-carbon metabolism does not play a role in fragility pathogenesis (Matteini and Belli 2001).

Effect of low folate. This is the first investigation into the behavioral aspects of *tg*-MTHFR mice exposed to a folate deficient diet. Wild type and heterozygous *tg*-MTHFR mice fed a low folate diet for 6 months caused a decrease in forelimb grip strength in both male and female mice but the affect was not worsened in heterozygous MTHFR mice fed a low folate diet (Figure 48). The other behavioral parameters assessed were not influenced by folate status except for folate deficient female heterozygous *tg*-MTHFR mice, which had a decrease in vertical plane entries (Figure 49-54). Interestingly, folate status impacted *tg*-MTHFR mice and C57BL/6J mice differently, specifically in grip strength and open field behavior. Low folate negatively impacted grip strength in *tg*-MTHFR whereas; a folate deficient diet for 3 months had a positive effect in aged C57BL/6J mice. In addition, open field behavior was negatively affected by folate status in C57BL/6J mice and was not in *tg*-MTHFR mice. The differing effects of folate in these two mouse models may be attributed to the variation in the two strains of mice that were tested. Other investigators have reported that different strains of mice can perform differently on behavioral test (Crawley 2000). Secondly, the *tg*-MTHFR mice were treated with the diet for twice the amount of time as the C57BL/6J mice, thus the effect of low folate on grip strength may just take longer to induce.

Summary

Gender and age in a mouse are major determinants of its behavior. Thus extreme care should be taken to ensure all experimental groups contain equal numbers of males and females and that the mice are closely match in age. This will ensure that the study results reflect the experimental conditions. Open field behavior was reduced in mice fed a folate deficient diet. This observation is potentially due to anxiety and/or depression, which supports the connection between folate and psychiatric illnesses. In aged C57BL/6J and *tg*-MTHFR mice it appears that folate deficiency does not replicate the phenotype we see in the elderly, specifically that low serum folate levels are associated with a decline in cognitive testing scores. Most importantly low folate and folate deficient diets fed for 3 months to C57BL/6J mice and 6 months to *tg*-MTHFR mice did not result in any statistically significant changes in spatial memory as assessed by MWM. However, mice on control diets performed poorly on the MWM test and this invariably reduces the sensitivity to detect significant changes.

CHAPTER SEVEN

General Conclusion

The work presented in this dissertation outlines the biological importance of folate metabolism and its role in maintaining normal CNS function. Epidemiological studies in the aging population have shown that folate deficiency can lead to depression, cognitive decline, and dementia (Morris et al. 2008; Bottiglieri 2005; Bottiglieri et al. 2000; Nelson et al. 2009). There has been renewed interest in this field of research over the last decade that has provided a better understanding of the clinical associations; however the biochemical and neurochemical basis is still not clearly defined. In order to study the mechanism(s) involved in folate deficiency induced CNS disorders it is important for researchers to develop a mouse model that closely replicates the metabolic and phenotypic changes seen in humans. This dissertation has focused on characterizing the neurochemical and behavioral changes in young and old folate deficient C57BL/6J mice. In addition, an aged *tg*-MTHFR mouse model was assessed because of the similarity to the MTHFR 677TT genotype, which is commonly over-represented in the human population.

The first aim of this study was to characterize the effect of age on metabolites of the methylation cycle in C57BL/6J mice sacrificed by CO_2 asphyxiation (Chapter 3). In humans plasma tHcy levels rise as folate concentrations decline with age (Clarke et al 1998; Miller 1999; Snowdon et al. 2000). This inverse relationship was not observed in this study. Old mice had 3-fold lower plasma tHcy levels compared to young mice with no differences in plasma 5-MTHF levels. It is probable that remethylation of Hcy to

methionine is up regulated in old mice, supported by evidence that mRNA levels for both MTR and MTRR are significantly increased in liver tissue from old mice compared to young mice. Furthermore, methylation reactions occurring in liver tissue are the major source of Hcy which is exported into the circulation (Refsum 1998). Plasma levels of SAM and SAH were also decreased in old mice, although the principal methylation metabolites, methionine, SAM and SAH were not affected by age in liver or brain tissue. Betaine was elevated approximately 3-fold in liver tissues from old mice probably due to decreased utilization supported by data showing that mRNA levels of BHMT were reduced in liver tissue from old mice. Generally, it appears that in C57BL/6J mice, age does not affect methylation metabolites, specifically in brain tissues. However, it is likely that age-related differences may be masked by post-mortem effects when mice are sacrificed by CO_2 asphyxiation. It was shown in chapter 4 that rapid sacrificing of mice by microwave radiation had a significant effect on SAM and SAH levels, as well as other metabolites related to pathways with a high metabolic turnover rate.

The second aim of this study was to determine the effect of folate deficiency on metabolites of the methylation cycle in young and old C57BL/6J mice, and old *tg*-MTHFR mice using two different sacrifice techniques (Chapter 4). The rationale for this study was based on previous studies showing that folate deficiency is more prevalent in an aged population compared to a younger population. Furthermore, the MTHFR 677TT genotype is moderately widespread in the human population, and leads to increased sensitivity to folate deficient related disorders, including depression and dementia (Lewis et al. 2006; Reif et al. 2005; Gilbody et al. 2007; Yoo et al. 2000). The hypothesis tested

was that old mice, specifically those with less MTHFR activity would be more susceptible to the effects of dietary folate deficiency.

Prior to studying the effects of folate deficiency, the effect of microwave radiation as a technique to sacrifice young and old C57BL/6J mice was evaluated. Data from old C57BL/6 mice showed elevated and variable levels of adenosine indicating that the microwave technique did not heat the brain tissue uniformly and therefore failed. It was also unfortunate that old *tg*-MTHFR mice were too large to be placed inside the microwave restraint holder and therefore no data using this technique could be obtained. For these reasons, only metabolite data from young C57BL/6 mice was used in the final analysis of microwave radiation.

In young C57BL/6J mice microwave radiation caused a significant decrease in SAM (28%), SAH (92%), and choline (81%), and a significant increase in betaine (48%) and the SAM/SAH ratio (89%) in brain tissue. The difference in the concentration of these metabolites between the two methods of sacrificing mice are likely owing to microwave radiation resulting in a rapid (< 0.9 second) heat inactivation of enzymes related to the methionine cycle, including methyltransferase enzymes. This is in contrast to the method of sacrificing by CO_2 asphyxiation where enzymes are metabolically active in the several minutes of time it takes to remove the tissues and either freeze or deproteinize. As a result data obtained by microwave radiation is suggested to give a more accurate measurement of the concentration *in vivo*, or as close as possible to the time of death. This is the first report of brain methylation metabolite concentrations from mice sacrificed by microwave radiation and therefore represents novel data. For the

studies involving folate deficient diets it was decided that microwave radiation would also be used as a method of sacrifice in addition to CO_2 asphyxiation in young mice.

As previously reported and replicated by this study, dietary folate deficiency led to an accumulation of plasma tHcy in both C57BL/6J and *tg*-MTHFR mice due to diminished remethylation to methionine (Fuso et al. 2008, Troen et al. 2008). Old mice fed a folate deficient diet had the highest level of tHcy, which was not statistically significant from young mice, fed the same diet. More importantly dietary folate deficiency resulted in a decrease in the SAM/SAH ratio in brain tissue, an effect that was independent from age, gender, mouse strain, or method of sacrifice (CO_2 versus microwave). This observation is consistent with previous studies of folate or B vitamin deficiency that used methods to sacrifice mice other than microwave radiation (Miller et al. 1994; Sontag et al. 2008; Troen et al. 2008; Fuso et al. 2008). It is important to emphasize that the methylation metabolite data presented in chapter 4 shows the cause for reduced SAM/SAH ratio in brain tissue from folate deficient mice differs depending on the method of sacrifice. In mice sacrificed by CO_2 asphyxiation brain SAH levels were increased, whereas SAM levels remained for the most part unchanged. In mice sacrificed by microwave radiation SAH levels remained either unchanged or mildly affected, whereas SAM levels were decreased. The data presented in this thesis establish that folate deficiency induced hypomethylation is likely to be the result of SAM deficiency, which limits methyl group availability, rather than increased SAH and feedback inhibition of methyltransferase activity. These findings are important in understanding the metabolic basis of folate deficiency induced CNS disorders and developing therapeutic interventions.

Folate deficiency and hypomethylation is implicated in the pathology of age-related cognitive decline and other dementias. The neuropathological hallmark of AD is the presence of neurofibrillary tangles and amyloid beta plaques. The expression and deposition of these neurotoxic proteins have been shown to be influenced by methylation. Neurofibrillary tangles develop when Tau protein is phosphorylated in a reaction that is predominantly catalyzed by glycogen synthase kinase 3 beta (GSK3B). Dephosphorylation of phospho-Tau occurs by the action of protein phosphatase 2A (PP2A). The amount of phospho-Tau that accumulates in brain tissue is the result of the balance in activity between GSK3B and PP2A. It has been shown that the activity of PP2A is dependent on the carboxymethylation of a critical amino acid residue (leucine 309) on the catalytic subunit C (Sontag et al. 1996). It was recently reported that mice reared for 2 months on a folate deficient diet had reduced PP2A substrate specificity, decreased methylation of the catalytic subunit C and increased phospho-Tau in several brain regions (Sontag et al. 2007, 2008). In this study mice were sacrificed by CO_2 asphyxiation and the hypomethylation of PP2A subunit C and increase in phospho-Tau was attributed to an increase in brain SAH concentrations. Currently brain tissue from the experiments presented in this dissertation are being tested for levels of phospho-Tau, methylated subunit C, and PP2A activity by Dr. Sontag (University of Newcastle, Australia) by Western blot analysis. The aim is to determine if similar changes in PP2A and phospho-Tau are present in mice sacrificed by microwave radiation.

In recent years researchers have focused on the relationship between methylation and the amyloid beta plaques present in AD patients. Amyloid beta is formed after sequential cleavage of the amyloid precursor protein (APP) by beta (BACE), and gamma

secretases. Presenilin 1 (PS1) is postulated to regulate APP processing by stabilizing the gamma secretase protease complex. Fuso and colleagues showed that in a culture medium devoid of folate and B_{12} SAM levels were reduced, which was associated with increased activity of PS1 and BACE; ultimately resulting in an increase in production of amyloid beta; however the addition of SAM was able to reverse these effects (Fuso et al. 2007). More recently in a folate, B_{12}, and B_6 deficient AD mouse model (TgCRND8) he reported that PS1, BACE, and amyloid beta deposition were all increased and that the mice had slight cognitive impairments prior to plaque formation (Fuso et al. 2008). Moreover, it was determine that the increase in PS1 was due to hypomethylation of the promoter region of the gene encoding PS1. More important was the finding that these effects in the AD mouse model could be overturned by exogenous SAM administration (Fuso et al. 2009).

Abnormal methylation of phospholipids is also postulated to contribute to the pathology of AD. A 10 week study in folate deficient young male rats reported that brain membrane content of the methylated phospholipid phosphatidylcholine (PC) was significantly depleted and performed worse on cognitive testing than the control group. In these deficient rats brain SAH levels were not altered but SAM levels were significantly diminished. These finding were reversed by the addition of methionine, the precursor of SAM (Troen et al. 2008). The studies lend further support that depletion of SAM due to folate deficiency is most likely responsible for hypomethylation rather than increased SAH. Furthermore, collectively, these studies are consistent with the hypothesis that altered methylation in association with folate deficiency may contribute to AD.

PC is acknowledged to be important in cognitive function because of its relationship to the neurotransmitter acetylcholine. Metabolic breakdown of PC generates choline. Choline is the precursor of the neurotransmitter acetylcholine, which is involved in learning and memory; and betaine, the alternate methyl donor in liver and kidney tissue. Decreased choline and acetylcholine levels have been reported in patients with cognitive decline (Nizri et al. 2007), but have rarely been investigated in animal models of folate deficiency due to the requirement for the use of microwave radiation as the method of sacrifice. In this study choline and acetylcholine were measured in young mice sacrificed by microwave radiation, and were significantly reduced in the mid-brain. It's likely that the effect of folate deficiency may be greater in the AD brain that is already deficient in these metabolites, and could accelerate the progression of the disease. Similarly, betaine levels were significantly decreased in all folate deficient mouse models presumably due to decreased availability of choline. To our knowledge this is the first study to measure betaine levels in brain tissue.

Cystathionine levels were significantly increased in brain tissue from mice reared on a folate deficient diet. The transsulfuration pathway is not intact in brain tissue (CSL is not present); therefore this organ relies on the uptake of cysteine from the circulation to support the synthesis of glutathione. It is not known if high levels of cystathionine are toxic to neuronal cells. In C57BL/6J mice reared on a folate deficient diet and heterozygous *tg*-MTHFR mice reared on a low folate diet the levels of cystathionine in brain tissue were 4- to 5-fold and 12- to 18-fold higher respectively compared to control mice. The highest concentration was present in the cerebellum in both strains of mice. Interestingly, *tg*-MTHFR deficient mice have been shown to have significant

neuropathological abnormalities and reduced cellular proliferation in the cerebellum (Chen et al. 2005).

Methionine concentrations were reduced by lack of folate in all mice except for old C57BL/6J mice; in these mice the levels were increased. One of the most important novel observations was that the effects of folate deficiency in brain and peripheral tissues, generally, were not exacerbated by age or heterozygosity for the MTHFR genotype with regards to metabolites of the methylation cycle.

The third aim of this study was to determine the effect of folate deficiency on neurotransmitter metabolism in young mice sacrificed by microwave radiation (Chapter 5). An earlier study has reported correlations between red cell folate and CSF BH_4 levels and also with CSF monoamine metabolites in depressed patients (Bottiglieri et al. 1992). BH_4 acts as the rate-limiting cofactor in the synthesis of serotonin and dopamine. The folate enzymes, MTHFR and DHFR, have been postulated to be involved in the salvage pathway of BH_4 regeneration (Figure 5, Chapter 1). Furthermore, MTHFR patients have been reported to have reduced levels of BH_4 and monoamine metabolites (Kaufman 1991), providing evidence for a role of folate in monoamine neurotransmitter metabolism. In this study it could not be confirmed that dietary folate deficiency affects the synthesis of BH_4 levels in the mouse brain. However, there is a large difference in BH_4 levels between species; plasma levels are 10-fold higher in mice than in humans, thereby may prove to be harder to deplete. A folate deficient mouse model has been shown to have lower hypothalamic serotonin turnover evident by a reduced 5-HIAA/5-HT ratio along with decreased caudate dopamine compared to control mice (Gospe et al. 1995). In this study young folate deficient mice also had reduced turnover of both

serotonin and dopamine in several brain regions, however, changes in the parent neurotransmitters were not observed. Although, this study was not able to confirm decreased synthesis of dopamine and serotonin due to lack of BH_4 production as reported in folate deficient humans it is feasible that a more severe folate deficient state may be needed to decrease BH_4 concentrations and impair monoamine neurotransmitter synthesis in mice.

The fourth aim of this study was to determine if the neurochemical changes related to folate deficiency in the mice used in this study are associated with behavioral deficits (Chapter 6). Gospe and co-workers reported that folate deficient mice exhibited impaired coordination as evidenced by their increase in food spillage (Gospe et al. 1995). In this study, folate deficiency did not induce a coordination deficit, which was tested by the rota rod task and swim speed in the Morris water maze task. However, exploratory activity was reduced in the young and old C57BL/6J folate deficient mice compared to control diet mice. Since folate deficiency did not cause any negative effects on locomotor ability, these findings may suggest that folate status can affect emotional aspects of open field behavior, such as anxiety, which could be tested by an elevated Plus Maze, or a light-dark exploration test.

A few folate deficient rodent studies have also found a negative correlation between folate status and spatial learning assessed by Morris water maze task (Bernardo et al. 2007; Troen et al. 2008; Lalonde et al. 2008). This study was unable to confirm this observation, additionally an age-related decline was not observed. However, the young and old C57BL/6J, and the old *tg*-MTHFR control diet mice used in this study appeared to be slightly impaired in the water maze task (latency above 20 sec.), thus spatial

memory impairments due to folate deficiency and/or age may have been masked by the performance of mice fed a control diet. Although, the reason for the impairment in the control groups is not known it may have been beneficial to perform a second or third test to evaluate memory, such as an elevated plus maze or a nose-hole poke test to confirm the results obtained by MWM. The original defense proposal included testing supplemented (5-MTHF) mice; however, due to the lack of cognitive impairments present in folate deficient mice this portion of the study was eliminated. Currently there are several transgenic AD mouse models available, most are based upon manipulating APP, PS1, apolipoprotein E, and/or Tau genes. Most of the models have cognitive decline by 6 months of age, it would be interesting to learn if lack of dietary folate would accelerate the progression of the disease and result in an earlier onset of cognitive decline. In relation to this it is encouraging that one group has reported that folic acid supplementation potentiated the effect of memantine on spatial learning in 8 month old Tg2576 transgenic AD mice and this was associated with decreased concentrations of amyloid beta fragments (Chen et al. 2010).

This is the first study to examine the effects of microwave radiation on methylation cycle metabolites and neurotransmitter metabolism, specifically in a folate deficient mouse model. Additionally, this was the first folate deficient study with an emphasis on methylation and behavior in an aged C57BL/6J and *tg*-MTHFR mouse models. This study was able to confirm many of the folate deficiency induce biochemical changes, but was unable to confirm deficits in spatial memory. More extensive behavioral tests in folate deficient mice would provide a better understanding of the effects of folate on cognitive function. Future studies in folate deficient mouse

models sacrificed by focused microwave radiation should prove valuable for further exploring the relationship between methylation, neurotransmitter metabolism, and CNS function.

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