IMPACT OF GENETIC AND NUTRITIONAL DISTURBANCES IN ONE-CARBON METABOLISM ON BRAIN FUNCTION AND STRUCTURE IN MICE

Nafisa M. Jadavji

Department of Human Genetics
McGill University
Montreal, Quebec, Canada

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Dedication

This thesis is dedicated to all the people who have been there for me through the good and bad.

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A wise person once said that it takes a village to raise a Ph.D. student and I completely agree. If it wasn't for my village, I would not have been able to complete this degree.

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Abstract

Homocystinuria can be caused by severe deficiency in either methylenetetrahydrofolate reductase (MTHFR) or methionine synthase reductase (MTRR). Patients present with neurological symptoms, some of which include developmental delays, mental retardation, motor abnormalities and brain atrophy. Biochemically patients have reduced enzyme activity, hyperhomocysteinemia and decreased levels of serum folic acid, methionine and *S*-adenosylmethionine (SAM). Mouse models for genetic deficiencies in MTHFR and MTRR have been developed to investigate the *in vivo* effects of these deficiencies. Young BALB/c *Mthfr*$^{-/-}$ mice have elevated levels of plasma homocysteine as well as decreased methylation and SAM levels in brain tissue. *Mtrr* is expressed in the developing neural tube and *Mtrr* expression is increased in brain tissue of MTRR-deficient mice. The first two objectives of this thesis will examine the brain function of MTHFR and MTRR in adult male mice.

Adult C57BL/6 *Mthfr*$^{-/-}$ male mice have elevated plasma homocysteine levels, impaired memory function along with changes in hippocampus, including morphology, global DNA hypomethylation, altered choline metabolites and increased levels of choline acetyltransferase (ChAT) and glucocorticoid receptor (GR). In the cerebellum of *Mthfr*$^{-/-}$ mice we confirmed morphological changes and increased apoptosis previously described in young BALB/c *Mthfr*$^{-/-}$ mice. Additionally, we identified changes in function, decreased volume and alterations in choline metabolites in the cerebellum.

Adult mice deficient in MTRR have mildly elevated plasma homocysteine levels, short-term memory impairments, gait and affective behavior changes along with reduced methylation in hippocampus and cerebellum and reduced hippocampal volume. Changes in choline metabolism in hippocampus, cerebellum, liver and plasma were also identified.

Maternal contributions of MTHFR, folic acid and choline during fetal and early neonatal brain development are essential. The last two objectives of this thesis will investigate the effects of maternal deficiencies in MTHFR, folic acid or choline on brain function and structure of 3-week-old *Mthfr*$^{+/+}$ offspring. Preliminary data suggests that maternal deficiencies in MTHFR can lead to impaired short-term memory and motor function in *Mthfr*$^{+/+}$ offspring. Elevated maternal plasma homocysteine and increased apoptosis in cerebellum and hippocampus of *Mthfr*$^{+/+}$ offspring may be responsible for these behavioural changes. Maternal folic acid (FADD) or choline (ChDD) deficient diets result in impaired short-term memory in *Mthfr*$^{+/+}$ offspring. Maternal ChDD results in impaired motor function, whereas maternal FADD changes affective behaviors in *Mthfr*$^{+/+}$ offspring. Both maternal FADD and ChDD result in increased apoptosis, alterations in choline metabolites and increased ChAT protein levels in the cerebellum and hippocampus of *Mthfr*$^{+/+}$ offspring.

These behavioural, morphological and biochemical results suggest that MTHFR, MTRR and maternal folate and choline are critical for brain development, maturation and maintenance of normal function.

Resumé

L'homocystinurie peut être due à un déficit des enzymes 5,10-méthylène tétrahydrofolate réductase (MTHFR) ou methionine synthase réductase (MTRR). Les patients peuvent manifester des symptômes neurologiques tels que retard mental, développement tardif, anomalies motrices et atrophie cérébrale. Au niveau biochimique, on observe une activité enzymatique réduite, une hyperhomocystéinémie et un taux sérologique moindre d'acide folique, de méthionine et de *S*-adénosylméthionine (SAM). Nous avons étudié des souris dont le gène *Mtrr* ou *Mthfr* est inactivé. Les jeunes souris BALB/c *Mthfr*$^{-/-}$ ont des taux élevés d'homocystéine plasmatique et moins de SAM ainsi qu'un taux de méthylation réduit dans le cerveau. *Mtrr* est exprimé dans le tube neural en développement et son taux de transcription augmente dans le cerveau de souris exprimant une version dysfonctionnelle du gène. Les deux premiers objectifs de cette thèse seront d'investiguer le rôle de MTHFR et MTRR dans le cerveau des souris C57BL/6 adultes mâles.

Les souris *Mthfr*$^{-/-}$ ont un taux plasmatique élevé d'homocystéine, des troubles de mémoire, des changements morphologiques de l'hippocampe, une hypométhylation de l'ADN et une altération des métabolites de la choline. L'expression de la choline acétyltransférase (ChAT) et du récepteur des glucocorticoïdes est augmentée. Nous avons noté un volume réduit du cervelet, des changements morphologiques et une fréquence plus élevée d'apoptose comme observé chez les jeunes souris BALB/c *Mthfr*$^{-/-}$. La distribution des métabolites de la choline est aussi altérée.

Pour les souris dont l'expression de MTRR est atténuée, on observe des concentrations plasmatiques d'homocystéine légèrement élevées, des troubles de mémoire à court-terme, une démarche modifiée, des changements de comportement accompagnés d'une hypométhylation de l'ADN dans l'hippocampe et le cervelet ainsi qu'un hippocampe à volume réduit. Des changements du métabolisme de la choline sont observés dans l'hippocampe, le cervelet, le foie et le plasma.

L'apport maternel en MTHFR, acide folique et choline est primordial pour le développement du cerveau durant la période fœtale et néonatale. Les derniers objectifs de cette thèse seront d'analyser les effets d'un déficit maternel en MTHFR, acide folique et choline sur le fonctionnement et la structure du cerveau des souris *Mthfr*$^{+/+}$ de la génération F1, âgées de 3 semaines. Un déficit maternel en MTHFR cause un trouble de mémoire à court terme et des problèmes moteurs chez ces souris F1. Ces changements peuvent être dus à la concentration plasmatique maternelle élevée en homocystéine et l'augmentation de l'apoptose dans le cervelet et l'hippocampe des souris F1.

Les mères dont l'apport en acide folique ou en choline est insuffisant ont des rejetons F1 *Mthfr*$^{+/+}$ ayant des troubles de mémoire à court terme. On observe aussi plus d'apoptose, une perturbation des métabolites de la choline et une augmentation de l'expression de ChAT dans le cervelet et l'hippocampe. La génération F1 *Mthfr*$^{+/+}$ présente des troubles moteurs ou des changements de comportement si l'alimentation de la mère était pauvre en choline ou en acide folique, respectivement.

Ces résultats suggèrent que MTHFR, MTRR, l'acide folique et la choline maternels sont primordiaux pour le développement du cerveau, sa maturation et son fonctionnement.

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Thesis Format

This thesis comprises 7 chapters. Chapter I is a review of the literature pertaining to this thesis. Chapter II is a description of all the materials and methods used in this thesis. Chapters III is a data chapter in the form it has been published in, excluding the materials and methods section. Chapters IV to VI are also data chapters. Chapters III to VI are linked by connecting text.

Contributions of Authors

The candidate designed the experiments, analyzed and interpreted the results and wrote the manuscript in chapter III and text in chapters IV, V and VI in collaboration with her supervisor, Dr. Rima Rozen.

In chapter III the candidate carried out all behavioural testing and related analysis. Qing Wu measured plasma homocysteine concentrations in 3-month-old mice. The candidate worked with Dr. Barry Bedell and Small Animal Imaging Laboratory (SAIL) to scan all animals using MRI. The candidate did all MRI volumetric analysis. The candidate sacrificed all animals and collected tissue and blood. The tissue morphological, apoptosis staining and Western blot experiments were done by the candidate. The candidate assisted in isolation of DNA from cerebellar and hippocampal tissue for TLC assay, the assay was completed by Liyuan Deng. For GR experiments in hippocampal tissue, the candidate designed the primers and conducted the mRNA experiments. The pyrosquencing experiments were done in collaboration with Dr. Daniel Leclerc. Dr. Leclerc designed and conducted GR CpG shore pyrosquencing experiments.

In chapter IV the candidate carried out all behavioural testing and related analysis. Qing Wu measured plasma homocysteine concentrations in 3-month-old mice. The candidate worked with Dr. Barry J. Bedell and SAIL to collect MRI scans. The candidate did all MRI volumetric analysis. The candidate sacrificed all animals and collected tissue and plasma. Xiao-ling Wang sectioned all brain tissue. The candidate stained and analyzed all tissue sections. The candidate assisted in isolation of DNA from cerebellar and hippocampal tissue for TLC assay, the assay was completed by Liyuan Deng. Primer design, mRNA and Western blot experiments were done by candidate.

In chapter V the candidate carried out all behavioural testing and related analysis. The candidate sacrificed all animals and collected tissue and plasma. Qing Wu measured plasma homocysteine concentrations in pregnant females and offspring. The candidate sectioned and stained the tissue for morphological and apoptosis analysis. Liyuan Deng isolated DNA from cerebellum and hippocampus and performed TLC assay. The candidate designed primers, conducted mRNA and Western blot experiments.

In chapter VI the candidate carried out all behavioural testing and related analysis, except pre-pulse inhibition testing was completed at Neurophenotyping Center of Douglas Mental Health University Institute. The candidate sacrificed all animals and collected tissue and blood. Qing Wu measured plasma homocysteine concentrations in offspring. The candidate sectioned, stained and analyzed brain tissue for morphological and apoptosis analysis. The candidate designed primers, and conducted mRNA and western blot experiments.

All choline metabolite measurements in cerebellar and hippocampal tissue as well as plasma were done by Olga Malysheva in Dr. Marie A. Caudill's laboratory at Cornell University.

Abbreviations

Ach	acetylcholine
AChe	acetylcholinesterase
Acetyl CoA	Acetyl coenzyme A
ApoA-I	apolipoprotein AI
ATP	adenosine triphosphate
BHMT	betaine homocysteine methyltransferase
CA1	cortical area 1
CA2	cortical area 2
CA3	cortical area 3
CBS	cystathionine-β-synthase
CD	control diet
ChAT	choline acetlytransferase
ChDD	choline deficient diet
CHDH	choline dehydrogenase
CNS	central nervous system
CSF	cereberospinal fluid
DHF	dihydrofolate
DHFR	dihyrdofolate reductase
DMG	dimethylglycine
DNMTs	DNA methyltransferases
dUTP	deoxyuridine triphosphate
dTTP	deoxythymidine triphosphate
FAD	flavin adenine dinucleotide
FADD	folic acid-deficient diet
FBP	folate binding protein
FR	folate receptors
GABA	gamma aminobutyric acid
IGL	internal granular layer
MCI	mild cognitive impairment
MeCP2	methyl CpG binding protein 2
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NMDA	N-Methyl-D-Aspartate
NTD	neural tube defect
PCFT	proton-coupled folate transporter
PEMT	phosphatidylethanolamine N-methyltransferase
PNS	pheripheral nervous system
PPI	prepulse inhibition
PtdCho	phosphatidylcholine
RCF1	reduced folate carrier 1
SAM	*S*-adenosylmethionine
SAH	*S*-adenosylhomocysteine

TLC	thin layer chromatography
TS	thymidylate synthase

Conventions

In this thesis, the names of genes and transcripts are italicised; uppercase for human (*i.e., MTHFR*), titlecase for mouse (*i.e., Mthfr*). The names of proteins are not italicised; both human and mouse proteins are uppercase (*i.e.,* MTHFR).

CHAPTER I: Literature Review

1.1 Folate

1.1.1 Folate structure

Folate is a water soluble B-vitamin that was first discovered in 1931 for its role in megaloblastic anemia during pregnancy [1]. In its stable form, folate consists of pteridine, *p*-aminobenzoic acid, and glutamic acid. For metabolic activity, folate can be reduced at positions 5, 6, 7, and 8 in the pteridine ring to produce tetrahydrofolate (THF) (**Figure 1.1)**. Folates can function as either coenzymes or cosubstrates by accepting, transferring or modifying one-carbon moieties. Folate derivatives differ in oxidation state of their pteridine ring at N^5 or N^{10} positions and the number of glutamate residues on the glutamic acid chain.

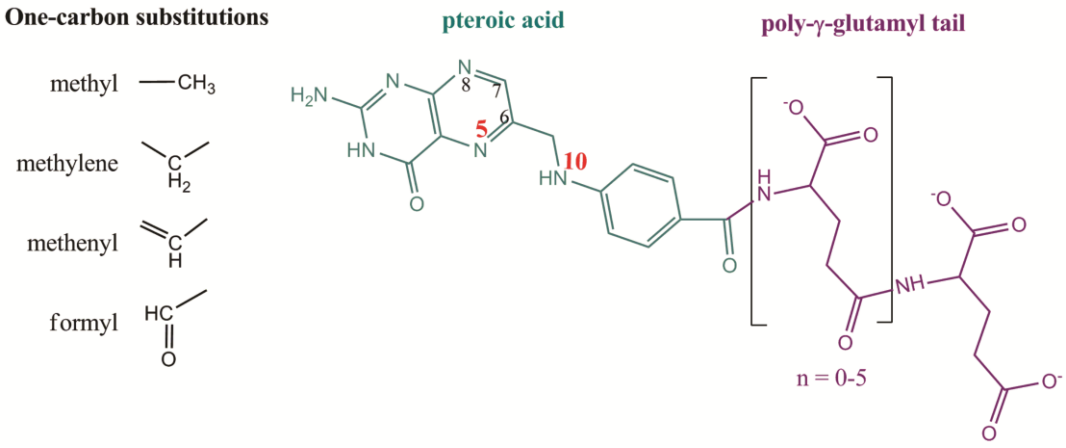

Figure 1.1 Structure of Folates

Folic acid, fully oxidized at positions 5, 6, 7, and 8. THF is reduced at positions 5, 6, 7, and 8. In THF, positions N^5 and N^{10} may be substituted with one carbon groups, shown on left, including methyl, methylene, methenyl and formyl. *Reproduced from Lawrance PhD thesis, 2007.*

1.1.2 Folate Intake

Humans can synthesize small amounts of folate in the intestinal microflora. It is then absorbed by cells within the large intestine [2-4]. However, intestinal folate synthesis does not generate sufficient amounts of folate in humans. Therefore, the majority of it is obtained from the diet or supplement use [2]. Foods high in folates include leafy greens, citruses, kidney and liver. Dietary forms are found in an unstable polyglutamate form (5-methylTHF) and must be hydrolyzed via folate hydrolase to the monoglutamate form before it can be absorbed using a carrier-mediated system in the jejunum of the small intestine [5-7].

Folate can also be obtained in a synthetic form, folic acid (pteroylmonoglutamic acid), through supplement use. This is already in a monoglutamate form and does not require hydrolysis to be absorbed by cells within the small intestine, where can be reduced to 5-methylTHF [8].

1.1.3 Folate absorption, transportation and distribution

1.1.3.1 Intestinal

Once folates are ingested, they are delivered to the duodenum from the stomach and are absorbed by the small intestine [9, 10]. Absorption in the small intestine occurs via a carrier-mediated process [11]. The reduced folate carrier (RFC1) is expressed in the brush border of the mouse intestine [12, 13]. The RFC1 is a transmembrane protein that allows for bi-directional transport of 5-methylTHF and folic acid [9]. However, RFC1 does not function optimally at a low pH, which is characteristic of the small intestine (pH=6) [10]. The proton-coupled folate transporter (PCFT) has been suggested to be the carrier involved in transportation

of folates and folic acid in the intestine [10] since it has a high affinity for folic acid and is efficient at a low pH [14].

1.1.3.2 Plasma

5-methylTHF monoglutamate is the main circulating form of folate found in plasma and serum. Approximately one-third of folates are unattached; the rest are bound to folate binding protein (FBP), which binds to reduced polyglutamates and oxidized monoglutamates, albumin, or other proteins [15, 16]. Erythrocytes do not metabolize folate and are reported to contain mostly 5-methylTHF polyglutamates [17, 18]. Folate status in erythrocytes does not fluctuate daily,whereas serum levels of folate do [16]. When folates enter circulation they are moved to the liver for storage as well as taken up by other tissues.

1.1.3.3 Cellular

Two types of transport exist for transporting folates into cells: carrier- and receptor-mediated processes [19]. The RCF1 carried-mediated process has been characterized for transport of folate and folic acid into cells [19]. In the mouse, RCF1 is expressed in most tissues including the small intestine, brain, spleen and kidney [20]. Furthermore, three human folate receptors (FR α, β, and γ) and the two related murine isoforms have been reported to play a role in bringing folates into the cell [21]. FR expression has been reported in the brain, liver, lungs, fibroblasts, placenta and ovaries [21]. These receptors mediate unidirectional flow of folate into the cell by endocytosis and have greater affinity for oxidized folates such as folic acid and are able to bind reduced forms of folates [21].

1.1.3.4 Gestation and lacation

During pregnancy, the demand for dietary folate is increased approximately by a factor of 5- to 10-fold. Increased intake of folate is needed for the development of the placenta and fetus, as well as maintaining normal function in maternal tissues. The demand for folate continues after birth through to lactation [21, 22].

The folate receptors play a role in transplancental folate transport during gestation. Maternal transfer of folate to the fetus occurs in two steps: first, 5-methylTHF is captured and bound to placental FR and secondly, maternal folates are transferred to the fetal circulation along a downhill concentration gradient [21].

Maternal milk provides an adequate amount of folate during early infancy which facilitates rapid growth during this period. Folates in human milk exist as either reduced polyglutmate [23] or as 5-methylTHF (20–40%) and are bound to folate binding protein, primarily FR-α [24, 25]. In order to ensure that the infant receives maximum folate during feeding, milk folates are approximately 5- to 10-fold higher than plasma folates [24, 26].

1.1.3.5 Central nervous system (CNS)

Only reduced forms of folate are transported into the brain [27], since dihydrofolate reductase (DHFR), an enzyme that reduces folic acid to THF, is not expressed in the brain. Reduced folates are transported into the brain via FRs. Both the blood brain barrier and choroid plexus contain RFC1, PCFT and FR-α receptors [10]. The choroid plexus is a structure in the ventricles of the brain producing cereberospinal fluid (CSF) and exporting of substrates and metabolites from the

CSF into the blood. 5-methylTHF is transported across the choroid plexus using the FR and then into the CSF [28, 29].

1.2 Folate metabolism

1.2.1 Overview

Folates are responsible for the transfer of one-carbon groups, production of nucleotides, and methionine. **Figure 1.2** is a simplified version of folate metabolism in the cytoplasm of cells. Upon entering the cell, folic acid is converted into 5,10-methylene-tetrahydrofolate (5,10-methyleneTHF) at which point the folic acid cycle bifurcates. 5,10-methyleneTHF can convert uracil to thymidine or it can be reduced in a reaction catalyzed by methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), a cytosolic enzyme. The product of the reduction reaction, 5-methylTHF, can remethylate homocysteine to methionine. This is catalyzed by methionine synthase (MTR) where methionine synthase reductase (MTRR) and vitamin B12 are activators of MTR. Methionine is then activated to produce *S*-adenosylmethionine (SAM), a universal methyl donor for many reactions, including DNA methylation [30] and neurotransmitter synthesis [31]. MTHFR provides the link between folate and homocysteine metabolism, as well as maintaining the balance between cellular methylation and nucleotide synthesis in cells [32].

A methylfolate trap occurs when there are increased levels of 5-methylTHF. Previous work has shown that this is a result of decreased levels ofvitamin B12 [33] or in MTR or MTRR deficiency [34] whereas there are reduced levels in MTHFR-mice [35].

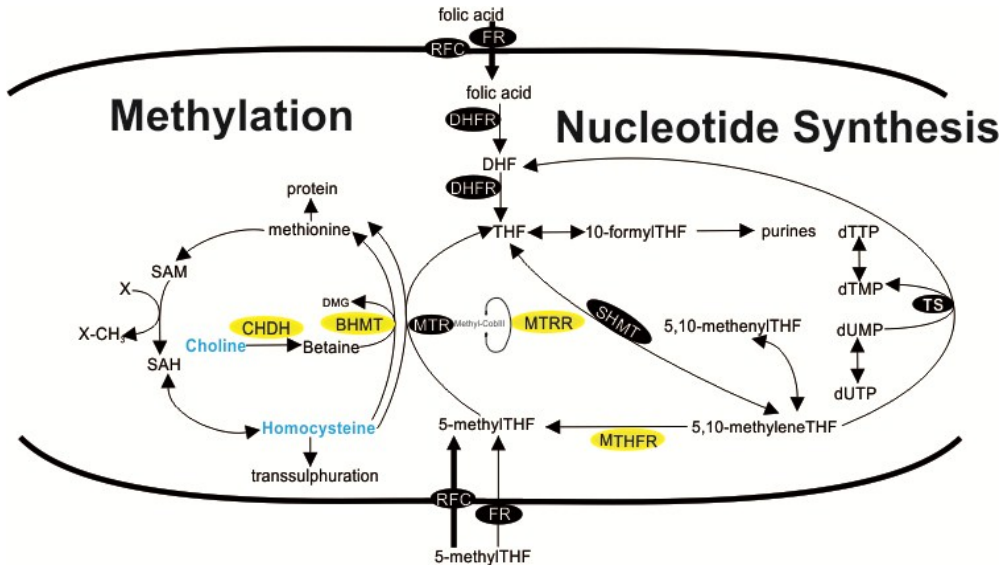

Figure 1.2 Folate Metabolic Pathway

Abbreviations: **BHMT** – betaine homocysteine methyltransferase; **CHDH** – choline dehydrogenase; **DHFR** – dihydrofolate reductase; **dTMP** – deoxythymidine monophosphate; **dTTP** – deoxythymidine triphosphate; **dUMP –** deoxyuridine monophosphate; **dUTP** – deoxyuridine triphosphate; **FR** – folate receptor; **MTHFR** – methylenetetrahydrofolate reductase; **MTR** – methionine synthase; **MTRR –** methionine synthase reductase; **RFC** – reduced folate carrier; **SAM** – *S*-adenosylmethionine; **SAH** *S*-adenosylhomocysteine; **TS** – thymidylate synthase. *Reproduced and modified from Lawrance PhD thesis, 2007*.

1.2.2 Nucleotide synthesis

Nucleotides are the basic building blocks that form RNA or DNA and play an important role in cellular metabolism. Nucleotides can be synthesized *de novo* or recycled using salvage pathways. Nucleotides are categorized into purines and pyrimidines, based on which nucleoside is attached to the sugar and phosphate backbone. Folates are involved in the *de novo* synthesis of purines and production of thymidylate, a pyrimidine. Carbon groups from 10-formylTHF assist in

assembly of the purine ring. In the production of thymidylate, the enzyme thymidylate synthase (TS) transfers a methyl group from 5-methyleneTHF to deoxyuridylate monophosphate (dUMP) to form deoxythymidylate monophosphate (dTMP) and DHF.

1.2.3 Homocysteine, *S*-adenosylmethionine and methylation reactions

Homocysteine is a non-protein forming sulfur-containing amino acid. The demethylation of methionine generates homocysteine [36]. Homocysteine can be remethylated back to methionine by 5-methylTHF, a product of the MTHFR reduction reaction [37], or by choline dehydrogenase (CHDH), betaine homocysteine methyltransferase (BHMT) and betaine, a metabolite of choline, in the liver and kidney [38]. Alternatively, a transsulphuration reaction using the vitamin B_6-dependent enzyme cystathionine-β-synthase (CBS) transfers a sulfur molecule from homocysteine to serine to form cystathionine and then cysteine [36, 39].

SAM is a global methyl donor and plays an important role in a number of diverse reactions including creatine and phosphatidylcholine synthesis (reference #37), but also plays a role in neurotransmitter synthesis [31, 37]and DNA methylation [30, 40]. SAM synthesis consists of SAM synthetase-mediated transfer of the adenosyl portion of adenosine triphosphate (ATP) to methionine and the remaining tripolyphosphate portion of ATP is hydrolyzed [30]. *S*-adenosylhomocysteine (SAH) is formed after the donation of a methyl group of SAM to a methyl acceptor, facilitated by methylase. Subsequently, SAH is hydrolyzed to adenosyl and homocysteine [30].

Epigenetic control of gene expression can occur through DNA methylation [30, 41], which consists of adding a methyl group by DNA methyltransferase (DNMTs) to the carbon-5 position of a cytosine on pyrimidine ring, resulting in 5-methylcytosine [41]. In mammals, 5-methylcystosines are present in 5'-CpG-3' and are located in CpG-rich regions, referred to as CpG islands [42, 43]. Approximately 60–90% of CpGs are methylated [30, 44] and 50–60% of all genes contain CpG islands [44, 45]. CpG islands compromise 1–2% of the genome and are between 200 base pairs to several kilo base (kb) pairs in length. CpG islands are associated with 5' ends of housekeeping genes and many tissue specific genes as well as 3' end of some tissue specific genes [45].

1.2.4. Methylenetetrahydrofolate reductase

MTHFR is a critical enzyme in folate metabolism; it regulates the distribution of methyl groups for nucleotide synthesis and methylation reactions (**Figure 1.2**). It requires a reducing agent, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), and a cofactor, flavin adenine dinucleotide (FAD). 5,10-methyleneTHF can provide methyl groups for nucleotide synthesis or can be reduced by MTHFR. The reduction reaction yields 5-methylTHF monoglutamate, the main circulatory form of folate. 5-methylTHF can donate a methyl group to remethylate homocysteine to methioine which can subsequently be activated to SAM, a universal methyl donor for intracellular reactions [30]. Activity of MTHFR can be inhibited by dihydrofolate (DHF) [46] and SAM [47, 48]. Post-transcriptional modification of MTHFR such as, phosphorylation, results in reduced enzyme activity and increased susceptibility for inhibition by SAM [49].

MTHFR was first cloned in 1994 [32] and has two promoter regions. There is 90% identity between human and mouse coding sequences. The human gene maps to 1p36.3 [32] and the mouse gene has been localized to the distal portion of chromosome 4 [50] . Characterization of both human and mouse *MTHFR/Mthfr* has revealed a complex structure with multiple levels of regulation. The first cDNA fragment cloned was a 2.2kb sequence comprised of 11 exons resulting in an active enzyme of 70kDa [51, 52]. Alternative splicing and/or polyadenylation can generate 3 *MTHFR* mRNAs of 2.8, 7.2 and 9.8 kb in humans and 2 mRNA transcripts of 3.2 and 7.5 kb in mice [53]. Furthermore, alternative splicing at the 5' end of the mammalian MTHFR gene results in two protein isoforms of the enzyme with a molecular weight of 70 kDa and 77 kDa respectively [54]. MTHFR is ubiquitously expressed throughout the body, with the highest expression reported in the testes, kidney, brain and colon, and lowest expression reported in the liver [54, 55].

1.2.5 Methionine synthase reductase

Methionine synthase (MTR) catalyzes the transfer of a methyl group from 5-methylTHF to homocysteine creating tetrahydrofolate and methionine [56]. Methionine synthase reductase (MTRR, E.C. 2.1.1.135) is essential in the reactivation of the cobalamin cofactor of MTR when is becomes oxidized to cobalt (II) via NADPH reduction reaction. MTRR, a flavoprotein, was cloned in 1998 [57] and is classified as a housekeeping gene. Conservation of several areas between the *MTRR* gene, *C. elegans* and rat cytochrome P450 reductase gene has been reported [57]. The human *MTRR* gene has been localized to chromosome 5p15.2-15.3 and is approximately 34 kb in length.

1.3 Choline

1.3.1 Choline structure and intake

Choline was first discovered in 1862. It is a quaternary amine, consisting of three methyl groups covalently attached to the nitrogen atom of ethanolamine (**Figure 1.3**) [58].

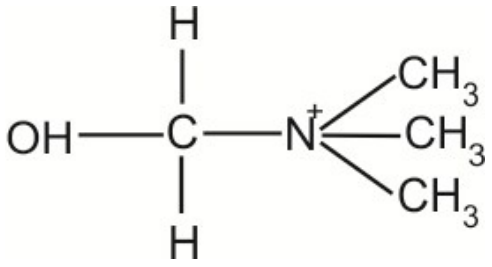

Figure 1.3 Chemical structure of choline

Modified from [59].

While choline can be synthesized in the liver, the majority of choline and its metabolite betaine are obtained from the diet [60]. Choline is found in foods like eggs, beef, pork, liver, soybean, and wheat germ, and betaine in wheat bran, wheat germ and spinach. In 1998 choline was added to the essential nutrient list in the United States [61].

1.3.2 Choline absorption, distribution and transportation

Choline is absorbed in the intestine, but can also be metabolized before entering the gut. In the intestine, bacteria can catabolize free choline into betaine to generate methylamines. Any remaining free choline is absorbed by the intestine via a carrier mediated transport system. In addition, pancreatic as well as intestinal enzymes can hydrolyze dietary phosphatidylcholine (PtdCho) to generate free choline, which is transported to the liver [58].

Choline is transported into tissues by diffusion and mediated transport. However, in the liver, kidney, mammary gland, placenta and brain, a carrier mechanism transports free choline [58]. The rate at which choline is transported in the brain is proportional to serum choline concentration [58]. Betaine, a metabolite of choline, is transported into the brain via transporters. The primary transporter is a GABA transporter, GAT2, which is primarly expressed in the cortex and cerebellum[62].

1.3.3 Choline Metabolism

Choline is the precursor of various metabolites (**Figure 1.4**). Intracellular choline phosphorylation, oxidation and acetylation are cell and tissue specific. PtdCho is a phospholipid which can be synthesized *de novo* from phosphatidylethanolamine, a reaction catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT). PtdCho accounts for 95% of the total choline pool and the remaining comprises of choline, phosphocholine, glycerophosphocholine, cytidine 5-diphosphochoine, and acetylcholine [59]. In the liver and kidney, choline is oxidized to betaine. In mitochondria, choline is converted to betaine via choline dehydrogenase (CHDH). Along with betaine homocysteine methyltransferase (BHMT), betaine can provide a methyl group to remethylate homocysteine to methionine and generate dimethylglycine (DMG) in the liver and kidney [38]. Acetylcholine (Ach) synthesis occurs in neurons both in the peripheral (PNS) and central nervous systems (CNS) and requires choline, acetyl coenzyme A (Acetyl CoA), and choline acetlytransferase (ChAT) in the process.

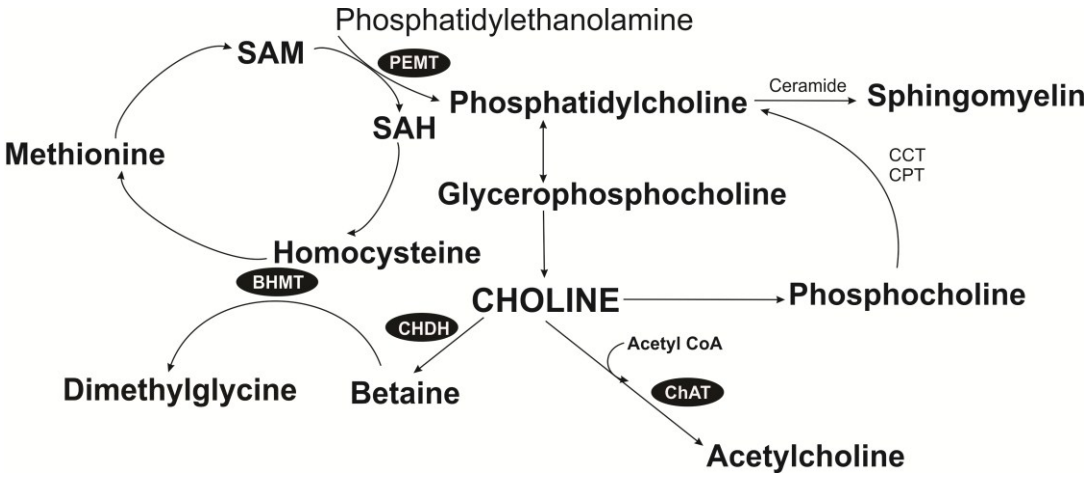

Figure 1.4 Choline Metabolism Pathway

Abbreviations: **Acetyl CoA** – acetyl coenzyme A; **BHMT** – betaine homosyteine methyltransferase; **ChAT** – choline acetyltransferase; **CHDH** – choline dehydrogenase; **DMG –** dimethylglycine**; PEMT** – phosphatidylethanolamine N-methyltransferase; **SAM** – *S*-adenosylmethionine; **SAH** – *S*-adenosylhomocysteine. Modified from [59], [63] and [38].

Choline and folate metabolism are tightly linked. In the liver and kidney, betaine (a metabolite of choline generated by CHDH) can provide an alternative methyl group for homocysteine remethylation [38, 64]. Human data [65] has shown that both MTHFR and folate status play a role in choline status. Work from the Rozen laboratory has shown that when folate-dependent remethylation is disrupted the choline-betaine pathway can serve as an alternative donor of methyl groups [66, 67]. The role of choline in the CNS is reviewed in **section 1.9.1**.

1.4 Dietary folate deficiency

In 1998, food fortification in both the US and Canada was implemented in order to reduce the incidence of neural tube defects (NTDs) [68]. However, folate

deficiencies are still prevalent in the human population [69]. Folate deficiency can be a result of reduced dietary folate intake or changes in folate metabolism, such as a polymorphism in MTHFR. Folate deficiencycan also be a result of chronic alcoholic use or medications including anti-convulsants [16] and L-3,4-dihydroxyphenylalanine (L-DOPA) [70]. Malabsorption as a result of celiac disease may also lead to folate deficiency [71].

In the cell, folate deficiency results in the inhibition of DNA synthesis which can alter cell division and affect cell proliferation and growth. This has been reported in erythrocytes and is referred to as megalobastic anemia [21, 72]. This condition results in abnormally large red blood cells that fail to divide because of reduced DNA synthesis causing the cell to remain in the growth phase of the cell cycle [16, 73]. Folate plays an important role in the developing and adult brain. The role of folate in the CNS is reviewed in **section 1.9.2.**

1.5 Hyperhomocysteinemia

Elevated plasma homocysteine or hyperhomocysteinemia is defined as fasting homocysteine levels exceeding 15 μmol/L. Hyperhomocysteinemia can be a result of dietary deficiencies of nutrients (e.g. vitamin B_{12}, choline or folic acid) or enzymes (e.g. MTHFR) involved in folate metabolism. Elevated levels of plasma homocysteine are strongly associated with increased risk of vascular disease [73]. Hyperhomocysteinemia has been linked to the development of many neuropathologies which will be discussed in **section 1.9.3**.

1.6 Genetic deficiencies in folate metabolism

1.6.1 Homocystinuria

Homocystinuria can result from severe deficiencies in MTHFR, MTR, and MTRR [74]. Biochemical features include hyperhomocysteinemia and

hypomethioninaemia [56]. Patients with homocystinuria present with symptoms during the first year of life. Clinical disease is characterized by developmental delay, seizures, learning difficulties, precocious atherosclerosis, and both arterial and venous thrombosis [75]. Treatment may include choline, betaine, and reduced dietary intake of methionine [76, 77].

1.6.2 Severe MTHFR deficiency

A severe MTHFR deficiency results in hypomethioninemia, decreased levels of circulating folate, and 0-20% residual enzyme activity [78]. Age of onset varies from neonatal period to adulthood [79]. Affected individuals present with various neuropathologies, such as developmental delays, severe brain atrophy, seizures, gait and motor abnormalities, all of which are possibly due to neuronal loss and demyelination during the neonatal or adolescent periods [76, 80, 81]. Patients that have been treated with betaine, a metabolite of choline, show improvements in growth and psychomotor development, as well as reduced levels of plasma homocysteine [76, 79, 80, 82]. The role of severe MTHFR deficiency in CNS is reviewed in **section 1.9.4.**

1.6.3 Severe MTRR deficiency

Affected individuals present with symptoms in early childhood [56]. Neurological symptoms include development delay, brain atrophy, hypotonia, white matter and electrophysiology abnormalities [83]. Patients also present with megaloblastic anemia [84, 85] and have hyperhomocysteinemia and hypomethioninaemia [56]. The role of severe MTRR deificency in CNS is reviewed in **section 1.9.5.**

1.6.4 Mild MTHFR deficiency

Many polymorphisms of *MTHFR* have been described in the human population. The best characterized polymorphism is a C→T transition of DNA position 677 resulting in a missense mutation causing an alanine to valine amino acid change [86]. Homozygosity for the 677 variant has been observed in 5-15% of North American and European populations [87]. The highest frequencies have been observed in some Hispanic groups and the lowest in Black populations [88]. Individuals with the 677TT genotype have 35-45% residual MTHFR enzyme activity, low plasma folate levels, and elevated plasma homocysteine when their folate status is low [51, 74, 87]. The 677 variant has been associated with increased risk for development of vascular disease, neuropathologies as well as NTDs [89, 90]. The role of mild MTHFR deficiency in the CNS is reviewed in **section 1.9.4.**

1.6.5 Mild MTRR deficiency

A common polymorphism described in the human population for MTRR is c.66A→G(p.I22M). Homozygosity for this polymorphism has been reported in approximately 25% of most populations [91]. Independently, the MTRR 66A→G variant does not affect plasma homocysteine levels [92]. However, plasma homocysteine levels are increased when the MTRR and MTHFR polymorphisms are combined or during vitamin B_{12} deficiency [91, 93]. Women with the 66GG genotype have increased risk of NTDs in their offspring [94]. Furthermore, studies have reported a greater risk for NTDs when the MTRR polymorphism and other vitamin B deficiencies are combined [91, 95]. The role of mild MTRR deficiency in CNS is reviewed in **section 1.9.5.**

1.7 Mouse models of genetics deficiencies in folate metabolism

1.7.1 MTHFR deficiency

To examine the *in vivo* effects of MTHFR deficiency on human disorders, development, etc., a mouse model was developed in both BALB/c and C57Bl/6 backgrounds [35, 96]. BALB/c *Mthfr*$^{-/-}$ mice have ~26% survival rate, whereas C57BL/6 have ~81% survival rate [96]. A part of this thesis will investigate behaviour and neurobiology of *Mthfr*$^{-/-}$ mice and therefore C57Bl/6 strain will be used for all experiments. Young BALB/c and C57Bl/6 *Mthfr*$^{-/-}$ mice have elevated plasma homocysteine levels and in brain tissue have decreased levels of SAM and global DNA hypomethylation [35, 96]. Furthermore, BALB/c *Mthfr*$^{-/-}$ have significantly reduced 5-methylTHF in total folate in both the liver and brain compared to wild-type mice [35]. Mice heterozygous (*Mthfr*$^{+/-}$) for MTHFR mimic the human 677TT genotype, both are phenotypically normal but have elevations in homocysteine and 50% residual MTHFR activity. *Mthfr*$^{-/-}$ mimic the severe MTHFR deficiency described in humans [35].

1.7.2 MTRR deficiency

The absence of MTRR may be embryonic lethal since it is expected to result in a complete lack of active methionine synthase (MTR), so a *Mtrr* gene trap model (*Mtrr*$^{gt(pGT1Lxf)XG334Byg}$, hereafter abbreviated as *Mtrr*^{gt}) was developed to study the *in vivo* effects of a methinione synthase deficiency [34]. The *Mtrr* gene contains 15 exons. A gene trap vector (pGT1Lxf) was inserted between exons 9 and 10, approximately 398bp downstream of exon 9. This resulted in the removal of exons 10 to 15 causing a disruption in critical electron transfer reactions and leading to decreased MTR activity [34]. The mouse model was maintained on the

C57Bl/6 background. *Mtrr*$^{gt/gt}$ mice have elevated plasma levels of homocysteine, lower levels of methionine and males show reduced growth compared *Mtrr*$^{+/+}$ mice.

1.8 Cerebellum and hippocampal development and function in mice

The development of the cerebellum in mice begins at approximately gestational day 10 [97]. The cerebellum is located posterior to the cerebral cortex and contains extensive folding referred to as foliations (**Figure 1.5A**). It receives input from joints, vestibular system and visual system, and projects back to motor control regions including the prefrontal and limbic cortical areas. The cerebellum contributes to motor function, balance, equilibrium, monitoring, correcting, coordinating, as well as mediating motor learning and skill acquisition [98]. It contains a number of distinct cell types which form the different layers. The granular layer contains granule cells which are the smallest and most densely packed; they are non-myelinated and release glutamate, an excitatory neurotransmitter in the brain. The Purkinje layer contains Purkinje cells, which are myleinated and release gamma aminobutyric acid (GABA), an inhibitory neurotransmitter. The molecular layer contains two types of inhibitory interneurons, basket and stellate cells, which also release GABA and are located near the Purkinje cell bodies [98].

The development of the hippocampus begins at gestational day 15 [97, 99]. The hippocampus is a subcortical, sausage-shaped structure located within the forebrain [100]. It is made up of two major U-shaped interlocking sectors, dentate gyrus, and Ammon's horn which consists of cortical area 1 (CA1), cortical area 2 (CA2) and cortical area 3 (CA3) regions (**Figure 1.5B**). The CA4 region is located on the boundary of the dentate gyrus [100, 101]. The dentate gyrus primarily

consists of granule and basket cells, whereas the CA1 and CA3 regions are made up of pyramidal cells with long apical dendrites [100]. The hippocampus is involved in cognitive function, specifically spatial learning and memory [101, 102]. It receives inputs from the entorhinal cortex, brain stem, septum, striatum, and prefontal cortex [100].

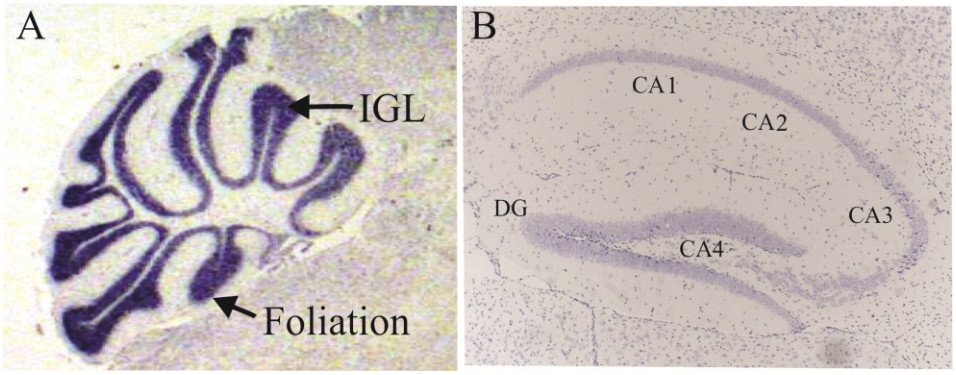

Figure 1.4 Mouse cerebellum and hippocampus morphology
A, cerebellum, internal granular layer (IGL) and foliation. B, hippocampus cortical area (CA1, CA2, CA3, CA4) and dentate gyrus (DG).

At embryonic day 9 neurogenesis is at the highest levels in the developing mouse brain [97]. Neurogenesis continues during gestation through to early post-natal development [103]. In the adult brain, neurogenesis in the brain is not widespread [104]. Interestingly, the cerebellum and hippocampus are structures in the brain in which neurogenesis continually occurs during adulthood [101, 104, 105].

1.9 Folic acid and choline metabolism in central nervous system

1.9.1 Role of Choline in Central Nervous System

Choline plays a critical role in brain function. Upon acetylation, it forms acetylcholine (Ach), a neurotransmitter present in both the CNS and PNS; upon

phosphorylation, it forms PtdCho, a component of the cell membrane. In the CNS, Ach is involved with synaptic plasticity, specifically in learning and short-term memory [106].

In a large population based study, choline concentrations have been reported to be negatively associated with anxiety symptoms which may be a result of disturbed cholinergic transmission [107]. Neural precursor cells grown in choline deficient medium are reported to have decreased expression of genes involved in cellular proliferation and DNA repair [108]. This could have detrimental effects during early brain development in terms of neuronal migration and differentiation, as well as during adulthood since support cells (e.g. glia) may not receive what they need to function.. Animals injected with a cholinergic neurotoxin, saporin, into the cerebellum displayed head tremors and ataxia [109]. In addition,, decreasing input from the medial septum to the hippocampus using Saporin resulted in animals with impaired learning and memory [110]. Choline supplementation has been reported to reverse some of the negative effects of folate deficiency on neurogenesis and increased apoptosis in fetal brains [111], as well as increase levels of nerve growth factor, involved in neuronal growth in the striatum [112]. Folate and choline metabolism are linked [38]; a study by [113] reported that BALB/c *Mthfr*$^{+/-}$ mice have decreased levels of Ach in brain tissues.

1.9.2 Folate Metabolism in Central Nervous System

Folic acid plays a central role in brain function during development as well as maintaining homeostasis [73]. One-carbon metabolism in the brain is involved in methylation, production of neurotransmitters and lipid metabolism synthesis

[114, 115]. The cerebellum and hippocampus have previously been reported to be affected by dietary folate deficiency [116, 117]. The folate concentration in the cerebellum and hippocampus is approximately 2.5 nmol folate/g, which is approximately 3-14% of that present in the liver [119].

Low plasma folate concentrations have been associated with development of cognitive impairments and dementia in the elderly population [120-122]. Decreased performance on episodic memory, namely object and word recall tests is observed in individuals who have low levels of folate in the blood [121]. Elevated plasma homocysteine levels are often observed in individuals with folate deficiency and have also been reported to be associated with impaired cognitive function [123]. Increasing folate intake may improve cognitive function, since high levels of plasma folate concentrations have previously been associated with better cognitive function [122]. For example, a recent study reports B-vitamins lowered homocysteine levels and slow cognitive decline in individuals with mild cognitive impairment (MCI) [124]. However, a meta-analysis of nine randomized trials reported that folic acid supplementation for three years had no effect on cognitive function [125].

In the brain, folates are necessary for biosynthesis of monoamine neurotransmitters including serotonin, epinephrine and dopamine [126]. Specifically, SAM donates methyl groups for the synthesis of these neurotransmitters [127]. Decreased levels of neurotransmitters have been reported in patients with depression [126]. A meta-analysis of eleven studies reported a significant association between folate status and risk of depression [128]. In

addition, a correlation between serum folate levels and duration of depressive episode was reported in patients with major depressive disorder [129].

Mice and rats on a folic acid deficient diet (FADD) have elevated levels of plasma homocysteine [116, 130]. In brain, these rodents have reduced levels of neurotransmitters [131], epigenetic alterations, such as global hypermethylation, alterations of histone patterns [132], decreased phosphatidylcholine levels [133] increased oxidative stress, apoptosis, and DNA damage [134] as well as decreased neurogenesis in the hippocampus [130]. Behavioural studies reported mice and rats on FADD have impaired cognitive function [113, 116, 133], increased anxiety, and increased activity [130, 135].

1.9.3 Hyperhomocysteinemia

Epidemiological studies have shown that folate deficiency and elevated levels of homocysteine are risk factors for development of dementia, Alzheimer's disease, and other neuropathologies [114]. *In vitro* data has shown that increased levels of homocysteine are associated with neuronal loss in the brain, possibly due to the excitatory properties of homocysteine [136, 137]. A study by [117] showed that high levels of homocysteine cause apoptosis in hippocampal neurons. The direct neurotoxic effect of homocysteine was examined by [138] and [139]; the authors of both studies reported that homocysteine induces cell death in the brain via the N-Methyl-D-Aspartate (NMDA) receptors. Glutamate is an excitatory neurotransmitter in the brain and homocysteine is thought to act as an agonist for a glutamate binding subunit on the NMDA receptor. The negative impact of homocysteine has also been observed in the behaviour of animals. Specifically,

elevated levels of homocysteine have been associated with cognitive impairments in spatial learning and memory using the Morris water maze [113, 116].

1.9.4 MTHFR deficiency

Epidemiological research has shown a link between a polymorphism of MTHFR at base pair 677C→T and increased risk for development of neural tube defects [140, 141] and neuropsychiatric disorders, such as depression [142], schizophrenia [143, 144]. Other neurological conditions linked to polymorphisms of MTHFR include decreased cognitive function [145, 146], dementia [147], Alzheimer's disease and Parkinson's disease [148]. However, all these studies require confirmation [143, 149].

DNA hypomethylation and decreased levels of SAM have been reported in whole brains of young BALB/c *Mthfr*$^{-/-}$. Changes in the cerebellum of young BALB/c *Mthfr*$^{-/-}$ mice have previously been reported, including decreased size, increased postnatal apoptosis, decreased external granular layer thickness and foliation compared to *Mthfr*$^{+/+}$ mice [35, 150]. In addition, changes in the expression of genes involved in intracellular calcium signalling have been described in whole brains of young BALB/c *Mthfr*$^{-/-}$ mice [151]. In addition, morphological changes in the hippocampus of mice [152] has been reported along with the expression of two MTHFR promoters [153]. Behaviourally, increased activity in the open field and decreased motor function on the rotarod task in BALB/c *Mthfr*$^{+/-}$ mice has been described [154]. Since the survival of BALB/c *Mthfr*$^{-/-}$ mice is lower compared to C57BL/6 mice [96], C57BL/6 mice are used in

this thesis. Additionall, sex differences in open field behaviour of BALB/c *Mthfr*$^{+/-}$ mice have previously been reported [154].

1.9.5 MTRR deficiency

MTRR expression in forebrain, midbrain, hindbrain, and neural tube has been described in day 9.5 old embryos [34]. Increased mRNA expression of *Mtrr* in the brain of *Mtrr*$^{gt/gt}$ mice has been reported. This up regulation may be a result of compensation for loss of MTRR in brain tissue [34]. There have been no reports of behavioural or neurobiochemical characterizations of MTRR-deficient mice to date.

1.10 Maternal deficiency of MTHFR, dietary folic acid and choline

Exposure to nutritional challenges during critical periods of early development can have negative effects on normal brain function and behaviour [106, 155, 156]. The brain and spinal cord begin as a flat plate that must be rolled up while edges must be joined to form a neural tube. This process fails to occur if folate is not present and results in NTDs [157]. In addition, the developing brain requires maternal contributions of essential nutrients and vitamins (e.g. folic acid and choline) for cell growth, differentiation, maturation, migration, synaptogenesis, and death [106, 158-161]. Therefore, an adequately nourished maternal environment is optimal to aid in the programming and limiting the number of potential perturbations to the developing fetal brain.

1.10.1 MTHFR Deficiency

In humans, a study reported that two-year-old children from *MTHFR* 677TT mothers had significantly reduced neurodevelopment compared to children from controls [162]. Risk of NTDs is elevated for maternal and fetal TT genotypes

by 50% and 80%, respectively [157]. In mouse studies, maternal MTHFR deficiency results in fetal growth retardation, including increased resorption rates, heart defects, number of delayed embryos as well as a significant decrease in embryonic crow-rump length and embryonic weight in offspring [163, 164].

1.10.2 Dietary Folic Acid Deficiency

Epidemiological studies have reported that a maternal FADD leads to increased NTDs [24, 165]. Low maternal folate intake during pregnancy has also been associated with increased risk of low birth weight and preterm delivery [72]. In terms of neurodevelopment, preliminary work by [166] has described that maternal FADD during early pregnancy may impair brain development which may increase hyperactivity in children however, more follow-up studies are required [166]. Since 1998, food fortification programs in both the United States of American and Canada have resulted in improved nutritional folate status as well as a decrease in the prevalence of NTDs [167].

In mice, previous studies have reported maternal FADD during gestation results in a longer gestational period [164, 168], decreased embryonic length and weight, increased resorption rates, higher number of delayed embryos, and heart defects [163], as well as increased anxiety in adult offspring [168]. Other studies in rodents have reported maternal FADD during gestational leads to increased apoptosis in the forebrain and impairments in learning, as well as a decrease of folate in brain of offspring [169-171].

1.10.3 Dietary choline deficiency

There is a high requirement of choline during pre- and postnatal neurodevelopment [59], especially for neural tube closure and hippocampal

development [63]. Maternal choline diet deficiency (ChDD) during embryonic development, results in increased heart defects in offspring [172]. Maternal ChDD, during embryonic days 11 to 17, has been reported to results in impaired memory function, sensory inhibition and neurbiological changes, including increased apoptosis and decreased DNA methylation in offspring [160]. Choline supplementation improves memory function as well as increases neurogenesis in the hippocampus of offspring [173-175]. Altered immune function has been reported in ChDD mothers and may contribute to defects previously described in offspring from ChDD mothers [176]. A study in adult rodents exposed to gestational choline deficiency reports altered hippocampal long-term potentiation (LTP)[177].

1.11 Thesis rationale

Genetic deficiencies in folate metabolism have been reported to influence brain function and structure. Epidemiological research has linked polymorphisms in methylenetetrahydrofolate reductase (MTHFR) to the development of various neuropathologies. Furthermore, a maternal polymorphism in methionine synthase reductase (MTRR) results in increased neural tube defects in offspring. These studies provide evidence that deficiencies in MTHFR or MTRR may result in changes in brain function and structure. Using a previously generated knockout mouse model for MTHFR and a gene trap knockout mouse model for MTRR, this thesis will investigate the role of both enzymes on brain function and structure in adult mice.

Maternal contributions of folates (genetic or nutritional) have been extensively investigated because of the role that folates play in the closure of the

neural tube. Maternal choline levels are important for the developing brain, as choline plays a role in generation of acetylcholine, lipid metabolism, and hippocampal development. Using the previously generated MTHFR-deficient mouse model, this thesis will investigate the effects of a maternal MTHFR deficiency as well as maternal dietary deficiencies in folic acid or a choline on the brain function and structure in *Mthfr*$^{+/+}$ offspring.

Aim One - Evaluate impact of MTHFR deficiency on the brain and behaviour of 3-month-old mice.

In previous studies, *Mthfr*$^{-/-}$ mice were reported to have changes in neurobiology, which may impact brain function. In this thesis, adult mice will be tested on multiple behavioural tasks at 3-months of age to assess motor and cognitive function, as well as affective behaviours. Cerebellar and hippocampal tissue will be analyzed for more detailed morphological and biochemical changes. The effects of a mild and severe MTHFR deficiency on brain function and structure will be assessed in this chapter.

Aim Two - Assess the impact of MTRR deficiency on the brain and behaviour of 3-month-old mice.

Patients with a severe MTRR deficiency present with neuropathologies; however, the exact mechanism through which this occurs remains unknown. Furthermore, epidemiological research has suggested a link between mild-hyperhomocysteinmia and cognitive impairment along with the development of Alzheimer's disease. For this aim, MTRR-deficient mice with mildly elevated plasma homocysteine levels, in contrast to *Mthfr* knockout mice with much higher elevations, will be tested on behavioural tasks at 3-months of age to assess brain

function. Plasma and tissue will be collected after completion of behavioural testing. The goal of this chapter is to evaluate the effects of mild-hyperhomocysteinmia *in vivo* as well as provide insight into potential mechanisms through which an MTRR deficiency may affect brain function.

Aim Three - Investigate the impact of maternal MTHFR deficiency on brain folate metabolism and behaviour in offspring at 3-weeks of age.

Epidemiological and basic research has reported that a maternal genetic deficiency in MTHFR results in negative outcomes in offspring; however, the outcomes on brain function and structure have not yet been investigated. This chapter will examine the brain function and structure of *Mthfr*$^{+/+}$ offspring from MTHFR deficient mothers.

Aim Four - Investigate the impact of maternal dietary folic acid or choline deficiency on brain folate metabolism and behaviour in offspring at 3-weeks of age.

Adequate maternal levels of folic acid and choline during pregnancy have been described to play a vital role during development *in utero*. Maternal folic acid deficiencies result in offspring with neural tube defects, heart defects and other abnormalities, whereas maternal choline deficiencies during embryonic day 11 to 17 result in impaired hippocampal function in offspring. This section will examine maternal dietary folic acid or choline deficiency on brain function and structure in wild-type offspring.

CHAPTER II: Materials and Methods

2.1 Animal experimentation

Animal experimentation was approved by the Montreal Children's Hospital Animal Care Committee, according to the guidelines of the Canadian Council on Animal Care. Behavioral testing was conducted at the McGill University-Montreal Children's Hospital Research Institute or the Neurophenotyping Center of the Douglas Mental Health University Research Institute. For all studies male mice and multiple litters were used.

For chapter three, the generation and genotyping of *Mthfr* knockout mice have been previously described [35]. The *Mthfr* null allele had been backcrossed for 13 generations onto the C57Bl/6 (Charles River Laboratories, Senneville, Canada) genetic background [96]. Heterozygous matings were performed to obtain $Mthfr^{+/+}$, $Mthfr^{+/-}$ and $Mthfr^{-/-}$ mice. Mice were fed commercial mouse chow diet 5001 (Agribrands Purina, St. Hubert, Canada). They were sacrificed after completion of behavioral testing at 3 months for all experiments, except for terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL) experiments, when they were sacrificed at 3 weeks of age.

For chapter four, the generation and genotyping of *Mtrr* gene trap knockout mice have previously been described in [34]. The *Mtrr* gene trapped null allele has been backcrossed for 8 generations on the C57BL/6 (Charles River Laboratories, Sennevile, Canada) genetic background. Heterozygous matings were performed to obtain $Mtrr^{+/+}$, $Mtrr^{+/gt}$ and $Mtrr^{gt/gt}$ mice. Multiple litters were used for each genotype group in all experiments. Mice were fed commercial mouse chow diet 5001 (Agribrands Purina, St. Hubert, Canada). They were sacrificed after completion of behavioural testing at 3 months.

For chapter five and six, the generation and genotyping of *Mthfr*-deficient mice have been previously described [35]. Heterozygous matings were performed to obtain $Mthfr^{+/+}$ and $Mthfr^{+/-}$ mice. For chapter five, at weaning female $Mthfr^{+/+}$ or $Mthfr^{+/-}$ were placed on control diet (CD) (2 mg folic acid/kg diet, Harlan Teklad, Indianapolis, IN), for 6 weeks before mating and remained on diet throughout pregnancy and lactation. For chapter six, at weaning $Mthfr^{+/+}$ were place on control diet (CD) (2 mg folic acid/kg diet, Harlan Teklad, Indianapolis, IN), folic acid deficient diet (FADD) (0.3 mg folic acid/kg diet, Harlan Tekland, Inidanapolis, IN) or choline deficient diet (ChDD) (300mg choline bitrate/kg diet and 2 mg folic acid/kg diet), Harlan Tekland, Inidanapolis, IN) for 6 weeks before mating and remained on diet throughout pregnancy and lactation. Females were mated with $Mthfr^{+/-}$ males. Male $Mthfr^{+/+}$ offspring were tested on behavioural tasks at weaning age (3-weeks old) and then sacrificed.

2.2 Total plasma homocysteine

Cardiac blood was obtained at sacrifice from 3-week-old, 3-month-old mice and pregnant females, collected in potassium-EDTA tubes and centrifuged at 7,000xg for 7 min at 4°C to obtain plasma, which was frozen until anlaysis. Plasma total homocysteine was quantified with the A/C Portable Enzymatic Homocysteine Assay (A/C Diagnostics, San Diego, CA) and A/C Diagnostics Reader (A/C Diagnostics) according to manufacturer's instructions.

2.3 Brain tissue analyses

The left hemisphere of the brain was used to excise the hippocampus and cerebellum for methylation, gene expression and protein analyses. The right hemisphere was fixed in 4% paraformaldehyde and paraffin-embedded. Tissue was

sectioned in serial sagittal orientation at 5-μm and sections were stained with cresyl violet. The thickness of the pyramidal cell layer of the hippocampus was measured; 6 measurements were averaged within CA1 and CA3 regions and 6 measurements were averaged within the dentate gyrus. The thickness of the cerebellar internal granular layer (IGL) was measured as described [96, 150]; two measurements were averaged in the same region of the anterior lobe. The degree of foliation was determined by counting the number of clearly defined lobes. All sections represented the same level along the longitudinal axis, and measurements were performed by two investigators blinded to genotype groups.

2.4 Behavioral testing

2.4.1 Footprint pattern

The footprint pattern task is a standard test for gait measurements in rodents [178]. Mouse hind paws were dipped in black non-toxic, water-based paint. Mice were placed in a clear Plexiglas runway lined with white paper and were allowed to walk to the opposite end of the runway. Four parameters were measured: stride length, base of support, print length and paw rotation [179]. Five measures were taken per animal per parameter.

2.4.2 Ladder rung task

The ladder rung test measures skilled motor function in rodents [180]. The testing apparatus was composed of two Plexiglas walls. Each wall contained holes located at the bottom edge of the wall; the holes could be filled with metal bars. The entire apparatus was placed on top two standard mouse housing cages, and animals with different genotypes were tested randomly. The performance was

video-recorded from the side, with the camera positioned at a slight ventral angle so that all 4 limbs could be recorded at the same time [180].

All video recordings were analyzed frame-by-frame. Each step was scored according to the quality of limb placement as previously described in [180]. For analysis of foot placement accuracy, the number of errors in each session was counted. The error score was calculated from the total number of errors and the number of steps for each limb.

2.4.3 Open field

Open field is commonly used to measure affective behaviours (e.g. anxiety) in rodents[178]. The open field box, measuring 46 x 46 x 12 cm, was made of clear Plexiglas. The bottom of the box was divided into 16 equal squares. At the start of testing, individual animals were placed in the middle of the open field box and video recorded for 5 min. After testing was complete, the floor of the box was disinfected with 70% ethanol. Video recordings were scored for vertical activity, activity (total number of squares crossed), fields entered in center and outside squares, and % time spent in the center and outside squares [181].

2.4.4 Elevated plus maze

The elevated plus maze measures anxiety in mice [178] and ismade of wood covered with black enamel, consisted of four arms (30 X 5 cm) shaped in the form of a +, and was elevated 50 cm from the floor. Two opposite arms were enclosed by side and end walls (12 cm high), the latter two arms were open. The connecting (open) center area measured 5 x 5 cm. At the start of each test, animals were placed in the center area facing an open arm. Trials lasted 5 min and were

videotaped. The number of open and closed arm entries, as well as time spent in each arm, was recorded [182].

2.4.5 Novel object recognition test (NOR)

The NOR can be used to asses short- or long-term memory[178].Testing was performed in an open field arena constructed with opaque gray Plexiglas. The stimulus objects varied in shape, color and texture, but were similar in size. During the training session, two identical copies of Object 1 were positioned in the open field arena and the mouse was allowed to explore both objects for 10 mins [183]. After the completion of the training session, mice were tested for short-term (60 mins) object memory [184] in chapters III, IV, V and VI. Additionally, in chapter III another group of mice was tested for long-term (24 hr) object memory [185]. During this test trial, animals were presented with one copy of the familiar stimulus (i.e. Object 1) and one copy of the novel stimulus not previously observed for 5 min. The amount of time spent exploring the objects during the training and test session was recorded. Relative exploration time during the test trial was expressed as a discrimination index [D.I. = (time novel - time familiar)/(time novel + time familiar)]. A positive DI indicates more time is spent exploring a novel object, whereas a negative score indicates that more time is spent exploring a familiar object. A score of 0 indicates that equal time is spent with both objects [184].

2.4.6 Y-maze test

The y-maze test assesses short-term memory in rodents[178].The maze was made of gray plastic fiber. Each mouse, naive to the maze, was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries was recorded. Arm entry was considered to be completed

when the hind paws of the mouse had been completely placed in the arm; the number of entries per arm was recorded [186]. Alternation was defined as successive entries into 3 arms, on overlapping triplet sets. The percentage of alternations was calculated as the ratio of actual to possible alternations (defined as the total number of arms entered minus two), multiplied by 100 [187].

2.4.7 Prepulse Inhibition (PPI)

For chapter six, mice aged postnatal day 28 and 60 were tested on prepulse inhibition (PPI), which measures inhibition in animals after a prepulse and startling stimulus (pulse) [178]. PPI was measured in 6 startle chambers (San Diego Instruments, San Diego, CA) consisting each of Plexiglas tubes mounted on a Plexiglas base within a sound-attenuating chamber. A piezoelectric strain meter attached to the base transduced the startle response. Stabilimeter readings were rectified, digitized on a 4095 scale, and recorded by a computer. A speaker located in the ceiling of the sound attenuating chamber presented all acoustic stimuli and maintained a constant background noise level of 70 dB. Startle reactivity was assessed by exposing animals to a 30 msec, 120 dB acoustic stimulus alone. An average of fifty 1-msec readings, beginning at the onset of the startle stimulus, was used as the dependent variable.

Prepulse inhibition (PPI) of acoustic startle responses was measured by having the 120 dB startle stimulus preceded by a 30 msec prepulse stimulus, which terminated 70 msec before the onset of the startle stimulus. The intensity of the prepulse stimulus varied from between 3 and 15 dB above the background noise level in 3 dB increments. A test session consisted of placing the animals in the startle chamber for a 5-min acclimatization period after which they were exposed

to a total of 37 trials separated by variable inter-stimulus-intervals that averaged 15 sec. The first 2 initial trials were startle trials. Results of the very first 2 trials were discarded as animals generally over-react to them. Over the last 35 trials, animals were exposed to additional 10-startle trials, and to 5 trials at each of the 5 prepulse intensities. These trials were presented randomly, with the one restriction that no more than two trials of the same type could occur in succession. For data analysis, the average of the last 10-startle trials was taken as the measure of startle reactivity for each animal. We also averaged the 5 trials taken at each of the 5 prepulse intensities, and then expressed these values as a percentage of the average reactivity for the 10 startle trials, using the formula: [(startle-prepulse)/ startle] x 100.

2.5 Magnetic resonance imaging (MRI)

MRI was performed at the Small Animal Imaging Laboratory (SAIL) at the Montreal Neurological Institute. Mice were anesthetized with an induction dose of 3.5-4% isofluorane and secured in an MRI-compatible bed. All MRI studies were performed under ~1.5% isofluorane in medical air and animals were allowed to breathe spontaneously without mechanical ventilation. Respiration rate and body temperature were continuously monitored using an MR-compatible system (Small Animal Instruments Inc., Stony Brook, USA) and the temperature was maintained at 37 ± 0.2^{o}C throughout the study using a feedback-regulated, air-warming system (Small Animal Instruments Inc., Stony Brook, USA).

All MR images were obtained from a 7T Bruker Pharmascan system (Bruker Biospin, Ettlingen, Germany) using a 28-mm inner-diameter, quadrature volume resonator (RAPID MR International, Columbus, USA). The imaging

protocol consisted of acquisition of scout images for anatomical localization, shimming, and acquisition of anatomical images. The anatomical images were acquired using a balanced steady-state free precession (b-SSFP) sequence with the following parameters: matrix size = 128×128×64, field-of-view = 1.8 cm×1.8 cm×0.9 cm, spatial resolution = 140 μm×140 μm×140 μm, excitation flip angle = 30^{o}, TR = 5.2 ms, TE = 2.6 ms, 4 phase-cycles, and 4 averages. The total imaging session lasted approximately 1 h per animal.

Images were converted from DICOM to MINC image file format. Whole brain, hippocampus and cerebellum from 3-month-old male mice were manually segmented by 2 independent raters using the Display software package (Montreal Neurological Institute, Montreal, Canada), 3 to 4 animals were used per genotype group. The volumes were subsequently calculated for each of the labeled structures.

2.6 Global DNA methylation

Global DNA methylation in hippocampus and cerebellum was measured by thin layer chromatography (TLC) as previously described [188]. Briefly, 5 μg genomic DNA was digested with *MspI* (which digests both methylated and unmethylated CCGG sequences). The DNA was treated with calf intestinal alkaline phosphatase, end-labeled with [^{32}P]dATP, hydrolyzed with nuclease P1, spotted on a cellulose TLC plate and developed in isobutyric acid–water–ammonium hydroxide (66:33:1), which allowed for the separation of nucleotides, including 5-methylcytosine. The relative intensity of cytosine and 5-methylcytosine was quantified by phosphorimagery and data are presented as the percentage of methylated cytosines/(methylated cytosines + unmethylated cytosines).

2.7 Terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL)

Brain tissue from 3-week-old animals was stained using TUNEL reactions which were performed as described in [150]. Only strong-staining nuclei were counted in the hippocampus and cerebellum, at 400x magnification. Data are presented as the number of TUNEL positive cells per high power field (hpf), one hpf = 90.6mm^2. In 5 animals per genotype group, a total of the same 4 chosen fields per hippocampus, or 5 fields per cerebellum, were quantified by two investigators blinded to the genotype groups.

2.8 Quantitative real-time RT-PCR

RNA was extracted from ~15 mg frozen hippocampus using the RNeasy Lipid Tissue Mini kit (Qiagen, Toronto, Canada). cDNA synthesis and real-time reactions were performed as described [189]. Primers for glucocorticoid receptor (GR (*Nr3c1*); sense 5'-ATG GGA GAG ACC GAA ACA AA-3' and antisense 5'RT-PCR-TCC AGA AGC CGA AAG TCT GT-3'), (*Bhmt*; sense 5'- CTC ATG AAG GAG GGT TTG GA-3' and antisense 5'-AAT CCC TGT TTG CCA CAG TC-3'), choline dehydrogenase (*Chdh;* sense 5'- GGC TCT CCA AGG AGG CTT TT -3' and antisense 5'-GTC CTC TAG CTG CTG CCT GT-3'), methionine synthase (*Mtr*; sense 5'-GTC AAC AGC ATC AGC CTC AA-3' and antisense 5'AAA GCC ATA ACC ACC ACA GC-3), methionine synthase reductase (*Mtrr;* 5'- TTA TTG GGC TTG GGT GAC TC-3' and antisense 5'- TCC AAG CTC CTG AAG TCG TT-3') and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh;* sense 5'-CAT GGC CTT CCG TGT TCC TA-3' and antisense 5'-CCT GCT TCA

CCA CCT TCT TGA T-3') were designed using Primer 3 software. Target gene expression was normalized using *Gapdh*.

2.9 Western blotting

Protein extracts were prepared using a Polytron homogenizer. Fifteen milligrams frozen hippocampus or 30 mg frozen cerebellum were homogenized in RIPA buffer containing complete-mini protease inhibitor (Roche Diagnostics, Laval, Canada) and cleared by centrifugation. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Primary antibodies were: choline acetyltransferase (ChAT; Millipore, Billerica, USA), glucocorticoid receptor (GR; Santa Cruz Biotechnology, Santa Cruz, USA), GAPDH (Cell Signalling Technology, Boston, USA). Secondary antibodies were horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (GE Healthcare, Mississauga, Canada) and HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, USA), as appropriate. Detection was achieved using ECL Chemiluminescence Plus (GE Healthcare, Mississauga, Canada). Bands were quantified by densitometry using Quantity One 4.1.0 (Bio-Rad Laboratories, Mississauga, Canada) and normalized to GAPDH by two investigators blinded to genotype groups.

2.10 Measurements of choline metabolites

Hippocampal and cerebellar choline, betaine, and Ach were measured by the LC-MS method of [190]; phosphatidylcholine, glycerophosphocholine, phosphocholine and sphingomyelin levels were measured as described by [191] with modifications [65, 192]. The same metabolites were measured in plasma as mentioned above. Other metabolites measured in plasma included dimethyl glycine

and trimethylamine were measured using the LC-MS method of [190] and lysophophatidylcholine levels were measured as described by [191] with modifications [65, 192]. Ach is not present in plasma, therefore was not measured.

2.11 Pyrosequencing analysis of methylation

In chapter three, DNA was isolated from ~30 mg hippocampus using the DNeasy Blood & Tissue Kit (Qiagen, Toronto Canada). Sodium bisulfite treatment of DNA was performed with EpiTect Bisulfite Kit (Qiagen, Toronto, Canada). All reactions were performed as recommended by the manufacturer. After isolation, the bisulfite-treated DNA was stored at -20°C until further use. Primers for pyrosequencing analysis were designed using proprietary PyroMark Assay Design 2.0 software. Amplification of a 100bp amplicon for the GR gene was performed with the Pyromark PCR kit (Qiagen, Toronto, Canada), prior to pyrosequencing. The sequence for the modified forward primer was 5'-GAGTTTTAGAGGGGGTGATAGTTAG-3', and the biotinylated reverse primer was 5'-ACCCCTCTACTAAAATAACACAC-3'. The sequencing primer for CpG-1 to 8 of mouse GR exon 1_7 (orthologous to the rat sequence identified by [193] was 5'-LC-MS-GAGGGGGTGATAGTTA-3'. Because CpG island shores have recently been shown to regulate gene expression[193], we also investigated two CpG dinucleotides within the GR gene CpG island shore, reported by [194] as chr18:39614176-39614226 of the mm8 UCSC database. The sequence for the modified forward primer was 5'-TTGGAAGGGGTAGTTGTTAAAAAA-3', and the biotinylated reverse primer was 5'-CTACCATAAACTTCTTCCCTATACAA-3'. The sequencing primer for the first two CpG dinucleotides of the 140 bp amplicon was 5'-GGGGTAGTTGTTAAAAAAG-3'. The denatured single-

stranded PCR products were purified with Streptavidin Sepharose High Performance beads (GE Healthcare, Mississauga, Canada) for acting as templates in the pyrosequencing reactions. All reactions were performed as recommended by the manufacturer (Qiagen, Toronto, Canada). Pyrosequencing was performed on a PyroMark Q24 platform (Qiagen, Toronto, Canada) and data were analyzed with PyroMark Q24 software.

2.12 Statistical analysis

Statistical analysis was performed using GraphPad software package 5.01 (GraphPad Software, La Jolla, USA, 1995). For chapters three and four, one-factor analysis of variance (ANOVA) was used, followed by Tukey's post-hoc HSD (Honestly Significant Differences) test. The significant post-hoc data are reported, unless otherwise indicated. For chapters five and six, un-paired t-tests comparisons between groups was used. In all analyses, a p-value of less than or equal to 0.05 was considered significant. All data are presented as mean $\pm$ standard error of the mean (SEM).

CHAPTER III: Severe methylenetetrahydrofolate reductase deficiency in mice results in behavioral anomalies with morphological and biochemical changes in hippocampus

Nafisa M. Jadavji, Liyuan Deng, Daniel Leclerc, Olga Malysheva, Barry J. Bedell, Marie A. Caudill and Rima Rozen

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3.1 Abstract

The brain is particularly sensitive to folate metabolic disturbances, since methyl groups are critical for its functions. Methylenetetrahydrofolate reductase (MTHFR) generates the primary circulatory form of folate required for homocysteine remethylation to methionine. Neurological disturbances have been described in homocystinuria caused by severe MTHFR deficiency. The goal of this study was to determine if behavioral anomalies are present in severe *Mthfr*-deficient (*Mthfr*$^{-/-}$) mice and to identify neurobiological changes that could contribute to these anomalies. Adult male mice of 3 *Mthfr* genotypes (+/+, +/-, -/-) were tested on motor, anxiety, exploratory and cognitive tasks. Volumes (whole brain and hippocampus) and morphology, global DNA methylation, apoptosis, expression of choline acetyltransferase (ChAT) and glucocorticoid receptor (GR), and concentrations of choline metabolites were assessed in hippocampus. *Mthfr*$^{-/-}$ mice had impairments in motor function and in short- and long-term memory, increased exploratory behavior and decreased anxiety. They showed decreased whole brain and hippocampal volumes, reduced thickness of the pyramidal cell layer of CA1 and CA3, and increased apoptosis in hippocampus. There was a disturbance in choline metabolism as manifested by differences in acetylcholine, betaine or glycerophosphocholine concentrations, and by increased ChAT levels. *Mthfr*$^{-/-}$ mice also had increased GR mRNA and protein. Our study has revealed significant anomalies in affective behavior and impairments in memory of *Mthfr*$^{-/-}$ mice. We identified structural changes, increased apoptosis, altered choline metabolism and GR dysregulation in hippocampus. These findings, as well as some

similar observations in cerebellum, could contribute to the behavioral changes and suggest that choline is a critical metabolite in homocystinuria.

3.2 Introduction

Folates are important vitamins that transfer one-carbon units for several critical reactions. The brain is sensitive to the supply of methyl groups for such functions as neurotransmitter synthesis and membrane lipid metabolism. Folates are also required for metabolism of homocysteine, an amino acid known to be neurotoxic in brain [136]. Epidemiological studies have suggested that elevated levels of plasma homocysteine are risk factors for development of neuropathologies [114].

Methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), a key enzyme in the metabolism of folate and homocysteine, catalyzes the synthesis of 5-methyltetrahydrofolate (5-methylTHF), the main circulatory form of folate. 5-MethylTHF is used for remethylation of homocysteine to methionine and *S*-adenosylmethionine (SAM), a universal methyl donor [30]. In liver and kidney, the choline metabolite betaine can serve as an alternate methyl donor for homocysteine remethylation, particularly when folate-dependent remethylation is disturbed [38, 195].

Genetic deficiencies in MTHFR can result in homocystinuria. MTHFR mutations in homocystinuric patients are associated with low levels of enzyme activity (0-20%), marked hyperhomocysteinemia as well as decreased levels of serum folic acid, methionine and SAM [76, 78, 196]. Clinical features vary, but often include developmental delays, mental retardation, motor abnormalities, psychiatric problems, cerebral atrophy, demyelination, and thrombosis [76, 197].

To examine the *in vivo* effects of MTHFR deficiency, a mouse model for mild and severe MTHFR deficiency was developed in our laboratory [35]. *Mthfr*$^{-/-}$ mice are a model for homocystinuria whereas *Mthfr*$^{+/-}$ mice with modestly reduced enzyme activity serve as a model for the mild hyperhomocysteinemia associated with a common sequence variant at bp 677. In our initial report of this mouse model, *Mthfr*$^{-/-}$ mice, backcrossed onto a BALB/c background, showed a low survival rate (26.5%). Nonetheless, we reported that young BALB/c *Mthfr*$^{-/-}$ mice had disrupted cerebellar morphology, increased apoptosis in cerebellum, decreased levels of SAM, global hypomethylation in whole brain and some structural changes in hippocampus [35, 150, 152]. No behavioral characterization or neurobiochemical analysis of the hippocampus in *Mthfr*$^{-/-}$ mice has been performed. Backcrossing of *Mthfr*$^{-/-}$ mice onto a C57Bl/6 background has resulted in a much higher survival rate, 81% [96], and has allowed us to examine this C57Bl/6 *Mthfr*-deficient mouse model for extensive behavioral and neurobiological characterization. In the present study, we demonstrate changes in affective behavior and impairments in short- and long-term memory, which are associated with several morphological and biochemical disturbances in the hippocampus of *Mthfr*$^{-/-}$ mice. We also confirmed and extended some of our earlier findings in the cerebellum of mutant mice.

3.3 Results

Behavioral assessment in *Mthfr*-deficient mice

Altered gait and decreased motor function in Mthfr$^{-/-}$ mice

Assessment of footprint patterns revealed that *Mthfr*$^{-/-}$ mice had altered degree of rotation compared to *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice (**Figure 3.1A**, $p<0.05$).

No differences were observed in other gait measurements. In the ladder beam walking task, *Mthfr*$^{-/-}$ mice had a lower movement score than both *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice (**Figure 3.1B**, $p<0.01$). Furthermore, *Mthfr*$^{-/-}$ mice made more errors while crossing the ladder (**Figure 3.1C**) compared to *Mthfr*$^{+/+}$ ($p<0.05$) and *Mthfr*$^{+/-}$ mice ($p<0.01$).

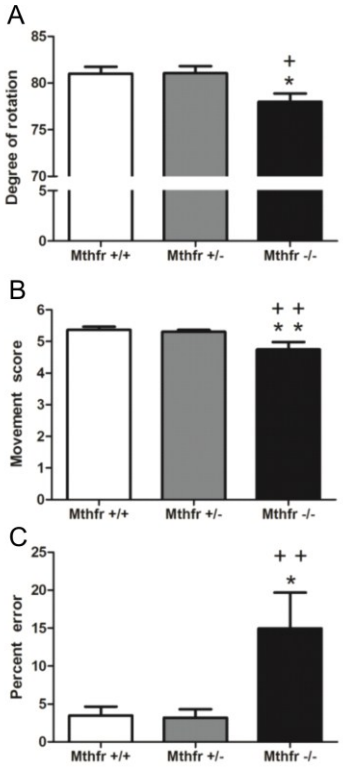

Figure 3.1 Effect of *Mthfr* genotype on gait (footprint pattern) and skilled motor function (ladder beam walking task) in 3-month-old male *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice.

A**,** degree of rotation; B, ladder beam movement score and C, % error. Bars represent mean $\pm$ standard error of 8 to 10 mice per group. *$p<0.05$, **$p<0.01$, post-hoc analysis, *Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/+}$. +$p<0.05$, ++$p<0.01$, post-hoc analysis, *Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/-}$.

***Mthfr*$^{-/-}$ mice exhibit more exploratory behavior and less anxiety**

Mthfr$^{-/-}$ animals had increased exploratory activity in the novel open field (**Figure 3.2A**) compared to *Mthfr*$^{+/+}$ ($p<0.01$) and *Mthfr*$^{+/-}$ mice ($p<0.05$). We also observed a trend for difference in time spent in the inside squares of the open field between genotype groups (ANOVA, $p=0.07$, data not shown); *Mthfr*$^{-/-}$ spent 18±3 % of the time, whereas *Mthfr*$^{+/+}$ spent 11±1 % in inside squares. The elevated plus maze findings were consistent with decreased anxiety for *Mthfr*$^{-/-}$ mice since they spent more time in the open arms of the maze compared to *Mthfr*$^{+/-}$ animals (**Figure 3.2B**, $p<0.05$).

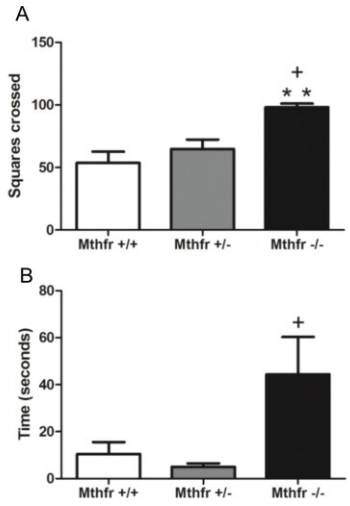

Figure 3.2 Effect of *Mthfr* genotype on open field activity and elevated plus maze tasks in 3-month-old male *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice.

A, open field activity (number of squares crossed); B, time spent in open arms of elevated plus maze. Bars represent mean ± standard error of 9 to 10 mice per group. **$p<0.01$, post-hoc analysis, *Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/+}$. +$p<0.05$, post-hoc analysis, *Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/-}$.

Cognitive function is disrupted in *Mthfr*$^{-/-}$ mice

We attempted to use the classic Morris water maze test for assessment of memory, but *Mthfr*$^{-/-}$ mice had impaired motor function and therefore this test was not suitable. Accordingly, we used the object recognition task to assess both short- and long-term memory, and the Y-maze to assess short-term memory.

The object recognition discrimination index for both one- and 24-hour retention tests showed, respectively, global short- and long-term memory impairment, in *Mthfr*$^{-/-}$ animals (**Figure 3.3A and 3.3B**). *Mthfr*$^{-/-}$ animals spent a larger portion of their exploration time with the familiar object compared to *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ animals ($p<0.01$).

The Y-maze test revealed a trend for differences in the number of spontaneous alternations due to genotype (ANOVA, $p=0.087$, data not shown). *Mthfr*$^{-/-}$ animals made significantly fewer alternations when compared to *Mthfr*$^{+/+}$ animals (t-test, $p=0.045$, data not shown).

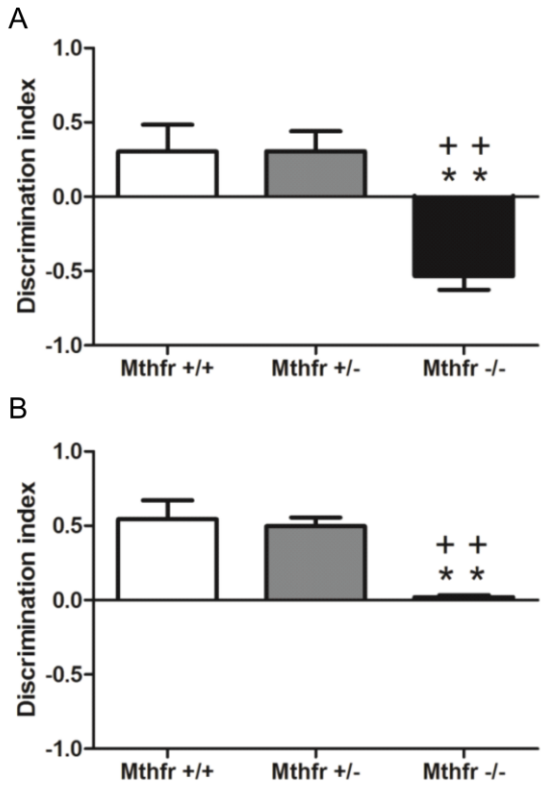

Figure 3.3. Effect of *Mthfr* genotype on novel object recognition (short- and long-term memory) in 3-month-old male *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and Mthfr$^{-/-}$ mice.

Novel object recognition, discrimination index after A, a one-hour and B, a 24-hour retention period. Bars represent mean ± standard error of 5 to 10 mice per group. **p<0.01, post-hoc analysis, *Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/+}$. ++p<0.01, post-hoc analysis, *Mthfr* $^{-/-}$ vs. *Mthfr* $^{+/-}$.

Decreased total brain weight and decreased whole brain and hippocampal volumes in *Mthfr*$^{-/-}$ animals

There were no differences in body weight between genotype groups. However, *Mthfr*$^{-/-}$ mice had significantly smaller brain weights as a percentage of body weight (1.07±0.06 %), compared to *Mthfr* $^{+/+}$ (1.6±0.09 %, p<0.001) and *Mthfr* $^{+/-}$ (1.4±0.6 %, p<0.01) mice. In addition, *Mthfr*$^{-/-}$ mice had significantly smaller whole brain volumes (**Figure 3.4A,** p<0. 01). Consequently, we calculated

hippocampal volume as a percentage of whole brain volume; *Mthfr*$^{-/-}$ mice had significantly smaller hippocampal volume (**Figure 3.4B**, $p<0.05$) compared to *Mthfr*$^{+/-}$ mice.

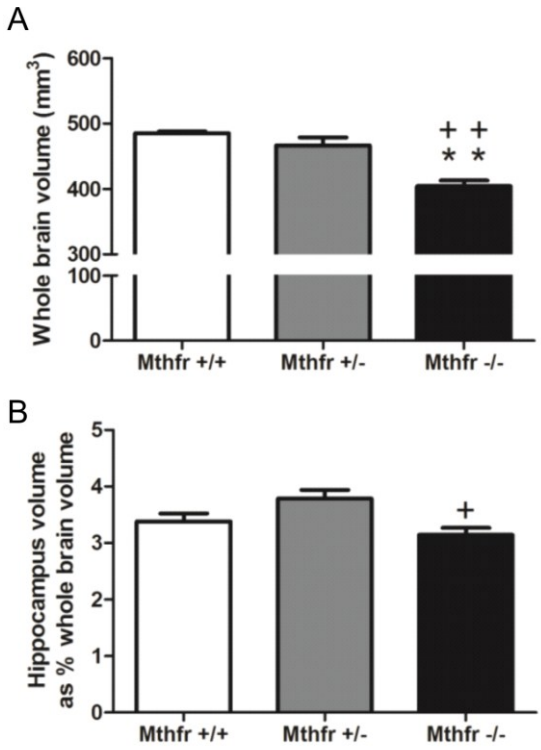

Figure 3.4. Effect of *Mthfr* genotype on MRI-defined volume of A, whole brain; B, hippocampus as a percentage of whole brain volume; of 3-month-old male *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and *Mthfr* $^{-/-}$ mice.

Bars represent mean ± standard error of 3 to 4 mice per group. **$p<0.01$, post-hoc analysis, *Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/+}$. +$p<0.05$, ++$p<0.01$, post-hoc analysis, *Mthfr*$^{-/-}$ vs *Mthfr*$^{+/-}$.

Differences in hippocampal morphology in *Mthfr*$^{-/-}$ mice

In *Mthfr*$^{-/-}$ animals, the CA1 and CA3 pyramidal cell layer in the hippocampus was significantly thinner when compared to *Mthfr*$^{+/+}$ mice (representative sections in **Figures 3.5A and 3.5B**, $p<0.05$). We also observed a

trend for difference in the thickness of the dentate gyrus between genotype groups (ANOVA, p=0.07).

Plasma homocysteine and global DNA methylation in hippocampus of Mthfr$^{-/-}$ mice

Plasma total homocysteine levels were significantly higher in *Mthfr*$^{-/-}$ mice when compared to *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice ($p<0.001$, data not shown), as previously reported [96]. Since high homocysteine can lead to disturbances in DNA methylation, we assessed global DNA methylation levels in hippocampus of the 3 genotype groups; however, there were no significant differences by ANOVA (data not shown).

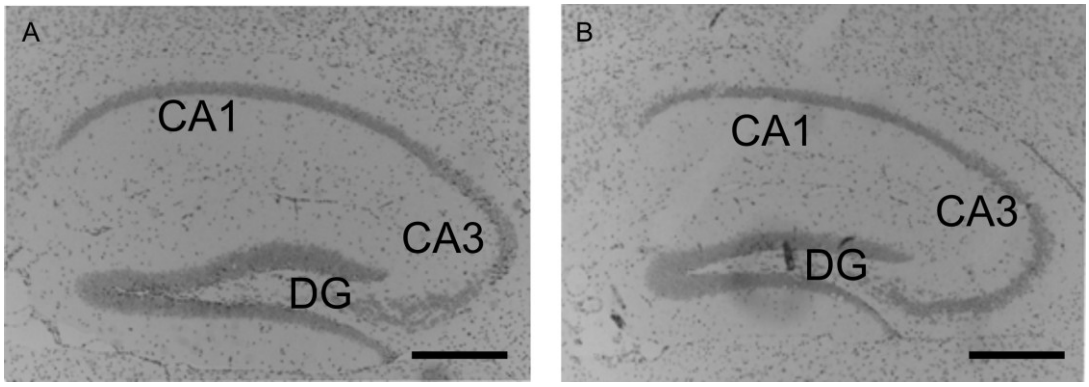

Figure 3.5 Cresyl violet stained brain tissue of *Mthfr*$^{+/+}$ and *Mthfr*$^{-/-}$ mice.
A-B, representative hippocampus sections of 3-month-old male *Mthfr*$^{+/+}$ and *Mthfr*$^{-/-}$ mice, respectively. All pictures at 10X magnification; scale bar = 200 µm. CA1, cortical area 1; CA3, cortical area 3; DG, dentate gyrus.

Apoptosis in hippocampus of *Mthfr*$^{-/-}$ animals

Normal 3-month-old mouse brain shows little or no apoptosis [103]. Therefore, we examined mouse brain at 3 weeks of age when there is a detectable

amount of apoptosis occurring. In the hippocampus, *Mthfr*$^{-/-}$ mice had significantly more TUNEL-positive cells (**Figure 3.6**) when compared to *Mthfr*$^{+/+}$ ($p<0.01$) and *Mthfr*$^{+/-}$ mice ($p<0.05$).

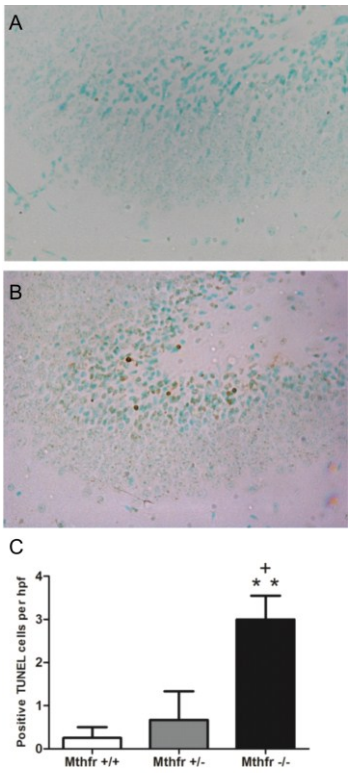

Figure 3.6 Effect of *Mthfr* genotype on hippocampal apoptosis in 3-week-old mice, as quantified by TUNEL-positive cells.

Representative TUNEL staining in dentate gyrus of hippocampus (A, B) of 3-week-old *Mthfr*$^{+/+}$ and *Mthfr*$^{-/-}$ mice, respectively. C, number of TUNEL-positive cells per high power field (hpf = 90.6mm^2) in hippocampus of male *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice. Bars represent mean $\pm$ standard error of 8 to 9 mice per group. **$p<0.01$, post-hoc analysis, *Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/+}$. +$p<0.05$, post-hoc analysis, *Mthfr*$^{-/-}$ vs *Mthfr*$^{+/-}$. All pictures at 400X magnification; scale bar = 50 μm.

Choline metabolites and ChAT protein levels in hippocampus of *Mthfr*$^{-/-}$ mice

There were no differences in free choline concentrations, but betaine concentrations were significantly lower in *Mthfr*$^{-/-}$ when compared to *Mthfr*$^{+/+}$ mice (**Figure 3.7A**, $p<0.01$). *Mthfr*$^{-/-}$ had significantly elevated glycerophosphocholine, compared to *Mthfr*$^{+/+}$ (**Figure 3.7B**, $p<0.05$) and *Mthfr*$^{+/-}$ ($p<0.001$) mice. We also observed a trend for the difference in acetylcholine concentrations between genotypes (**Figure 3.7C**, ANOVA, $p=0.059$). There were no differences in phosphatidylcholine, phosphocholine or sphingomyelin between genotype groups. As choline is not used as a methyl donor in the brain (i.e. *Bhmt* is not expressed in brain), these findings suggest that the oxidation of choline to betaine was curtailed in *Mthfr*$^{-/-}$ mice presumably to increase the availability of free choline for other metabolic pathways including acetylcholine and phosphatidylcholine biosynthesis.

To further examine the influence of *Mthfr* deficiency on acetylcholine biosynthesis, we measured immunoreactive protein levels of ChAT, which catalyzes synthesis of acetylcholine. As seen in Figure **3.7D**, immunoreactive protein levels of ChAT were significantly increased in *Mthfr*$^{-/-}$ ($p<0.05$) when compared to *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice. These findings suggest that up-regulation of acetylcholine synthesis may occur in mutant animals to replenish this critical neurotransmitter.

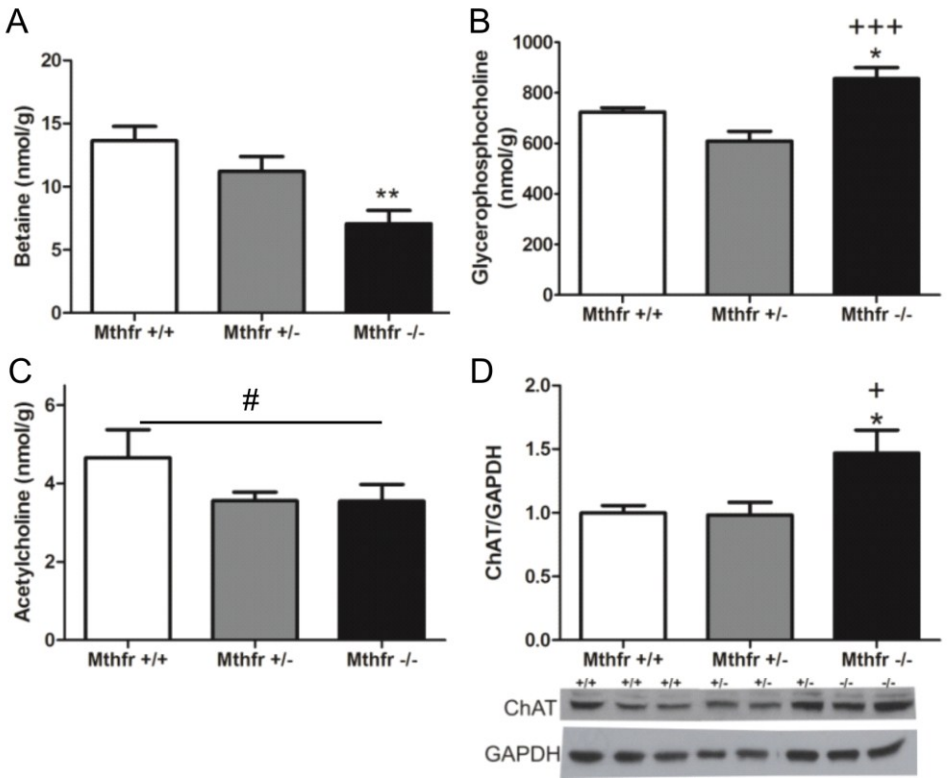

Figure 3. 7. Effect of *Mthfr* genotype on hippocampal choline metabolites.

A, betaine; B, glycerophosphocholine; C, acetylcholine and D, immunoreactive protein levels of choline acetyltransferase (ChAT) in 3-month-old male Mthfr+/+, Mthfr+/- and Mthfr-/- mice. Bars represent mean + standard error of 5 to 7 mice per group. (A, B, D) *p<0.05, **p<0.01 post-hoc analysis, $Mthfr^{-/-}$ vs. $Mthfr^{+/+}$. +p<0.05, +++p<0.001 post-hoc analysis, $Mthfr^{-/-}$ vs. $Mthfr^{+/-}$. C, #p=0.0599, one-factor ANOVA, no significant differences in post-hoc analysis. D, the panel below the graph depicts a representative Western blot

Glucocorticoid receptor (GR) in hippocampus

As mentioned above, we observed changes in anxiety levels of $Mthfr^{-/-}$ mice. The GRs in the hippocampus are involved in shutting down the stress response through the hypothalamic-pituitary-adrenal (HPA) axis [198]. Hippocampal expression of GR was investigated in *Mthfr*-deficient mice to determine whether the reduced anxiety was a result of GR changes.

Increased gene expression and protein levels of GR in $Mthfr^{-/-}$ animals

There was a significant difference in mRNA levels due to genotype (**Figure 3.8A**, ANOVA, $p<0.05$). Consistent with these results, *Mthfr*$^{-/-}$ mice had significantly higher protein levels of GR when compared to *Mthfr*$^{+/+}$ mice (**Figure 3.8B**, $p<0.05$).

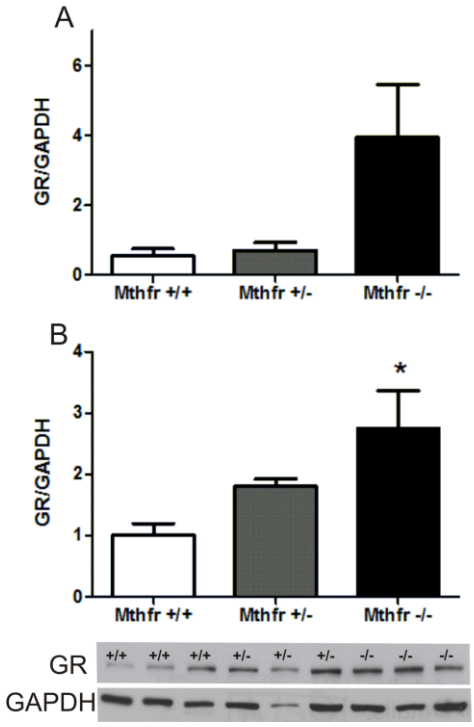

Figure 3. 8. Effect of Mthfr genotype on GR levels in hippocampus.
A, GR mRNA and B, immunoreactive protein levels in 3-month-old male *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice. Bars represent mean + standard error of 4 to 5 mice per group. A, #, $p<0.05$, one-factor ANOVA, no significant differences in post-hoc analysis. B, *$p<0.05$ post-hoc analysis, *Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/+}$. The panel below the graph depicts a representative Western blot.

Methylation within exon 1_7 of GR in hippocampus of *Mthfr*$^{-/-}$ animals

One group [193] had reported that increased GR mRNA expression could be modulated by decreased methylation within exon 1_7 of the GR. We investigated

methylation of the first eight CpG dinucleotides within exon 1_7 of the GR. Overall, there were very low levels of methylation (1-3%) in all eight CpG dinucleotides. At CpG dinucleotide 4, we observed a significantly decreased percent methylation between *Mthfr*$^{-/-}$ (0.97±0.04) compared to *Mthfr*$^{+/+}$ (1.19±0.07, t-test, p=0.05, data not shown). No changes in percent methylation were observed in the two tested CpG dinucleotides within the CpG island shore [194].

Morphology and neurobiochemical changes in cerebellum of *Mthfr*$^{-/-}$ mice

In the present study, we observed impaired skilled motor function and gait in *Mthfr*$^{-/-}$ mice. The cerebellum is reported to be involved the motor function [98]. In order to determine whether these impairments were consistent with previously reported cerebellar defects in young *Mthfr*$^{-/-}$ mice [35, 96, 150], we repeated some cerebellar analyses in the older mice of this study. We confirmed a thinner internal granular layer ($p<0.001$, data not shown) and decreased cerebellar foliation ($p<0.05$, data not shown) in *Mthfr*$^{-/-}$ mice, compared to both *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice. We also confirmed global DNA hypomethylation ($p<0.01$, data not shown) and increased apoptosis ($p<0.05$, data not shown) in the cerebellum of *Mthfr*$^{-/-}$ mice when compared to *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice.

We also performed some new analyses in cerebellum and observed significantly smaller cerebellar volume in *Mthfr*$^{-/-}$ mice (34±2.1 mm^3, $p<0.05$) when compared to *Mthfr*$^{+/+}$ (47.5±1.5 mm^3) and *Mthfr*$^{+/-}$ (47.8±1.2 mm^3) mice. Choline metabolite analysis revealed a trend for reduced levels of choline, (**Figure 3.9A**, ANOVA, p=0.09) between genotype groups and significantly reduced betaine in *Mthfr*$^{+/-}$ (Figure 2.9B, $p<0.05$) and *Mthfr*$^{-/-}$ (Figure 9B, $p<0.01$) when compared to *Mthfr*$^{+/+}$ mice. We also observed elevated levels of

glycerophosphocholine (**Figure 3.9C**, $p<0.05$) in *Mthfr*$^{-/-}$ when compared to *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mice as well as elevated immunoreactive protein levels of ChAT (**Figure 3.9D**, $p<0.05$) in *Mthfr*$^{-/-}$ when compared to *Mthfr*$^{+/+}$ mice.

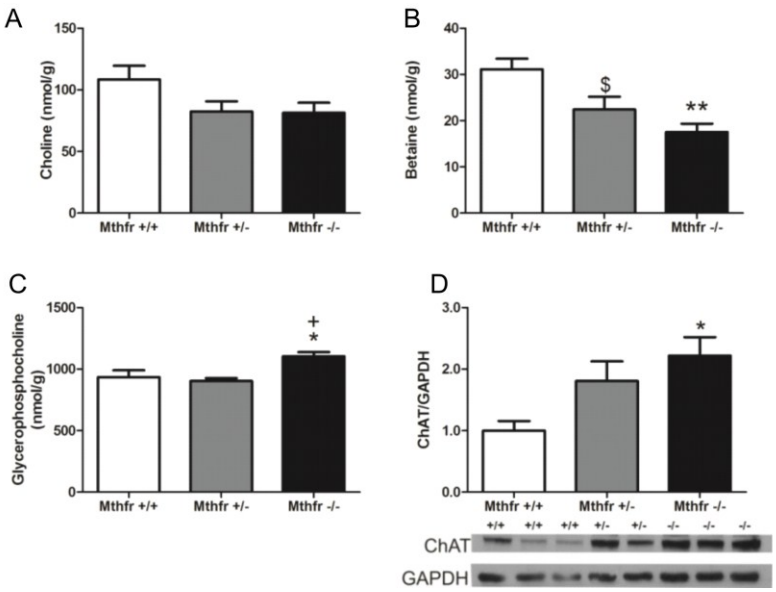

Figure 3.9 Effect of *Mthfr* genotype on cerebellar choline metabolites.

A, choline; B, betaine; C, glycerophosphocholine; D, immunoreactive protein levels of choline acetyltransferase (ChAT) in 3-month-old male *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ mice. Bars represent mean + standard error of 5 to 7 mice per group. A, one-factor ANOVA $p=0.09$, no significant difference in post-hoc analysis. (B-D) *$p<0.05$, **$p<0.01$ post-hoc analysis, *Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/+}$. +$p<0.05$, post-hoc analysis, *Mthfr*$^{-/-}$ vs. *Mthfr*$^{+/-}$. \$$p<0.05$, post-hoc analysis, *Mthfr*$^{+/-}$ vs. *Mthfr*$^{+/+}$. D, the panel below the graph depicts a representative Western blot.

3.4 Discussion

We identified impairments in motor and cognitive function as well as changes in affective behaviors in *Mthfr*$^{-/-}$ mice. Since the cerebellum is thought to be involved in motor function, we repeated some of our earlier analyses in

cerebellum [35, 96, 150] and confirmed some structural and biochemical changes; these changes include thinner internal granular layer, decreased foliation, and increased apoptosis in younger animals. In addition, we identified a decrease in volume and changes in choline metabolites. However, we concentrated on the hippocampus since this region is important in learning and memory [102] and has not been extensively studied in MTHFR deficiency.

Increased apoptosis and atrophy in *Mthfr*$^{-/-}$ mice

Apoptosis during postnatal development in brain is a common mechanism for removal of unnecessary neurons and optimization of synaptic connections [199]. However, excess apoptosis during early postnatal development may be unfavorable [200]. We observed increased apoptosis in hippocampus of *Mthfr*$^{-/-}$ mice, a finding that is consistent with the increased apoptosis in cerebellum [150]. The cerebellum has a 2-fold higher homocysteine concentration than any other brain region in mice [201]. Elevated levels of homocysteine have been shown to cause cell death through generation of excess calcium and reactive oxygen in vitro [138, 139].

We also found decreased whole brain, hippocampal and cerebellar volumes in *Mthfr*$^{-/-}$ mice. Elevated plasma homocysteine has been associated with whole brain [202] and hippocampal [203] atrophy in older human subjects. Furthermore, whole brain, cerebellar, and hippocampal atrophy have been proposed to be early markers in Alzheimer's disease [204, 205].

Disrupted choline metabolism in *Mthfr*$^{-/-}$ mice

Low levels of acetylcholine have been associated with cognitive impairments. Animals administered saporin, a neurotoxin exclusive to cholinergic

neurons, showed decreased performance on the Morris water maze task [206]. We observed significantly decreased concentrations of acetylcholine in hippocampus of *Mthfr*$^{-/-}$ mice. Consistent with this finding is the increased choline acetyltransferase levels in the hippocampus, as part of an attempt to restore acetylcholine pools [207]. Similar changes in choline acetyltransferase were found in cerebellum.

We also observed significantly decreased betaine in both hippocampus and cerebellum. The choline-betaine pathway serves as an alternative and important pathway for remethylation of homocysteine to methionine, especially when folate-dependent remethylation is disturbed [67, 208]. However, *Bhmt* is not expressed in brain. Thus the diminished concentrations of betaine in brain likely arose from a dampening of choline oxidation to betaine. In turn, this would spare free choline for conversion to acetylcholine and/or phosphatidylcholine via the CDP-choline pathway. The elevations in glycerophosphocholine, a catabolite produced during degradation of phosphatidylcholine to free choline, along with the enhanced expression of ChAT, imply that the hippocampus and cerebellum place a high priority on the biosynthesis of acetylcholine. Nonetheless, acetylcholine concentrations were diminished in hippocampus of mutant mice, a finding that highlights the integral role of methylTHF in the biosynthesis of acetylcholine. MethylTHF is required for the SAM-dependent biosynthesis of phosphatidylcholine, through phosphatidylethanolamine N-methyltransferase, which can subsequently be used to generate free choline and acetylcholine. Betaine is routinely used in treatment of homocystinuria; our work suggests that choline should also be considered, in light of the impact of MTHFR deficiency on choline metabolites in hippocampus and cerebellum.

Mthfr$^{-/-}$ mice have functional and morphological changes in hippocampus with increased GR expression

We had observed some structural changes in hippocampus of young _Mthfr_$^{-/-}$ mice on the BALB/c background [152], and, more recently, we reported expression of the two MTHFR promoters in hippocampus of newborn and adult mice [153]. Experimental and clinical work has demonstrated that the hippocampus is involved in spatial learning and memory [102]. In the present study, we observed that _Mthfr_$^{-/-}$ animals had significant impairments in short- and long-term memory using the object recognition task, which is a hippocampal-dependent learning task [209]. Furthermore, we observed a trend for decreased alternations in the Y-maze, which indicates a decreased working memory [186]. _Mthfr_$^{-/-}$ animals had significantly reduced thickness in the CA1 and CA3 regions, with a similar trend in the dentate gyrus. Decreased volume and increased apoptosis in hippocampus of _Mthfr_$^{-/-}$ animals during early postnatal development, as discussed above, may have led to the reduced number of cells in the hippocampus which could result in impaired connectivity between regions and consequent impairments in learning and memory [210].

Although the cerebellum has been reported to be involved in learning and memory, specifically in motor learning [211], we believe that the short- and long-term memory impairments in _Mthfr_$^{-/-}$ animals are more likely due to the changes observed in hippocampus. Furthermore, the memory impairments observed in _Mthfr_$^{-/-}$ mice are not a result of their motor impairment, since both the novel object recognition and y-maze tasks are choice measures [184, 187].

The hippocampus is enriched with the glucocorticoid receptors [212]. It has been suggested that GRs in the hippocampus are involved in shutting down the stress response, via the HPA axis [198]. Overexpression of the GR has been reported to increase HPA axis feedback regulation that may result in stress resistance in mice [213]. We observed decreased anxiety in *Mthfr*$^{-/-}$ mice along with increased GR protein and mRNA levels in the hippocampus. It is possible that the increased GR levels led to the decreased anxiety in our mouse model. GR has also been shown to have neuroprotective effects [214] and the increased oxidative stress created by hyperhomocysteinemia may have resulted in GR overexpression to limit neuronal damage.

Increased expression of GR in rat hippocampus has been reported to be regulated by methylation of exon 1_7 [193]. We investigated the first 8 CpG dinucleotides within the homologous region in mice using pyrosequencing, which enabled us to quantify methylation at each CpG dinucleotide. We observed very low levels of methylation (1–3%) across all 8 CpG dinucleotides, in contrast to the 5–70% methylation observed by others [193], and found a small, but statistically significant, decrease in methylation at CpG dinucleotide 4 in mutant mice. However, it is not clear whether such a small change is biologically significant. Other groups have shown increased mRNA expression of GR in their respective experimental models, but have been unable to link methylation of exon 1_7 to their expression results [215-217]. We cannot exclude the possibility that other regions of the GR promoter have methylation changes or that GR overexpression in our mice is not related to methylation changes.

In summary, we have observed significant changes in motor function, memory and affective behavior in *Mthfr*$^{-/-}$ mice which may be attributed to the neurobiological changes observed in the hippocampus and cerebellum. These changes may be due, at least in part, to the impact of hyperhomocysteinemia on choline metabolism or apoptosis. The increased expression of the glucocorticoid receptor in these mice is intriguing and requires further study to identify the link between this important transcriptional regulator and folate or homocysteine metabolism.

3.5 Acknowledgements

We would like to acknowledge the generous input of Dr. Joseph Rochford (McGill University, Douglas Mental Health University Institute) for assistance with the behavioral tests and for comments on the manuscript. We also thank Leonie Mikael, Qing Wu, Xiao-ling Wang and Eve-Marie Charbonneau for technical assistance. This work was supported by the Canadian Institutes of Health Research (MOP-43232). NMJ was a recipient of the Charles Banting and Frederick Best Canadian Institutes of Health Research Graduate Scholarship.

CONNECTING TEXT – Chapters III-IV

Homocystinuria can be a result of deficiencies in enzymes involved in folate and homocysteine metabolism, including MTHFR [78]. Patients present with neuropathologies, including seizures, gait abnormalities and developmental delays [76]. To examine the *in vivo* effects MTHFR deficiency, a mouse model for mild and severe MTHFR deficiency was developed in our laboratory [35]. *Mthfr*$^{-/-}$ mice are an animal model for homocystinuria. In chapter III we identified changes in hippocampal and cerebellar function of *Mthfr*$^{-/-}$ along with morphological and biochemical changes within the hippocampus. Choline metabolism in the hippocampus and cerebellum was significantly altered in these mice. These data highlight the importance of adequate one-carbon groups in hippocampal and cerebellar function and reiterate that folate and choline metabolisms are linked. In addition, they bring to light the possibility of treating patients with choline to help improve neurological symptoms associated with homocystinuria induced by a severe MTHFR deficiency.

Homocystinuria can also be caused by a severe deficiency in MTRR [56]. Patients present with neurological symptoms including brain atrophy, seizures, abnormal electrophysiology and developmental delays [83]. A mouse model was developed to study the *in vivo* effects of MTRR-deficiency [34]. A complete knockout results in embryonic lethality, therefore a gene-trap disruption in the *Mtrr* gene was used and these mice have elevated levels of plasma homocysteine [34]. Chapter IV investigates the effect of MTRR-deficiency on brain function and structure.

CHAPTER IV: Methionine synthase reductase deficiency in mice results in mild hyperhomocysteinemia, short-term memory impairments, biochemical changes in hippocampus and altered choline metabolism

4.1 Abstract

Elevated levels of homocysteine are a risk factor for brain atrophy, cognitive impairment and dementia. Methionine synthase, with methionine synthase reductase (MTRR) as activator, catalyzes the remethylation of homocysteine to methionine. A severe deficiency in MTRR results in homocystinuria. A common polymorphism in MTRR at bp 66 (A→G) has been reported in human populations. Mice with a gene trap for the *Mtrr* gene can serve as a model for the aforementioned human MTRR deficiencies. The purpose of this study was to investigate the role of MTRR deficiency on hippocampal function of MTRR-deficient mice. Male mice of 3 genotypes ($Mtrr^{+/+}$, $Mtrr^{+/gt}$ and $Mtrr^{gt/gt}$) were tested on two short-term memory tasks, object recognition and the y-maze. In the hippocampus, we assessed MRI-derived volume, global DNA methylation and expression of choline acetyltransferase (ChAT). Concentrations of some choline metabolites in hippocampal tissue and plasma were measured. $Mtrr^{gt/gt}$ mice had mild hyperhomocysteinemia and exhibited impairments in short-term memory. These mice had reduced hippocampal volume and reduced global DNA methylation. Choline metabolite analysis in the hippocampus revealed decreased levels of choline, betaine and acetylcholine with increased protein levels of ChAT in $Mtrr^{gt/gt}$ mice. In the liver of $Mtrr^{gt/gt}$ mice there were decreased levels expression of *Chdh* and *Bhmt* along with elevated levels of betaine in plasma. Our results suggest that mild hyperhomocysteinemia caused by MTRR deficiency leads changes in hippocampal function and disturbances in choline metabolism within the hippocampus, plasma and liver. This study highlights the negative impact of

mild hyperhomocysteinemia on memory, hippocampal function and choline metabolism in an animal model.

4.2 Introduction

Mildly-elevated levels of plasma homocysteine have been associated with neuodegeneration, specifically impaired cognitive function, as well as increased risk for development of Alzheimer's disease and dementia [218-220]. Since the prevalence of neurodegeneration is growing each year [220], it is important to understand the mechanism through which homocysteine impairs brain function. Folate, a B-vitamin, is involved in generation of one carbon groups that decrease homocysteine levels.

Methionine synthase, with methionine synthase reductase (MTRR) as activator, catalyzes the remethylation of homocysteine to methionine [57]. In order for optimal activity of MTR, it is dependent on co-factor (B_{12}) and methioine synthase reductase (MTRR, E.C. 2.1.1.135). Homozygosity for a common polymorphism in the MTRR gene, c.66A→G (p.I22M) has been reported in approximately 25% of North American and European populations [91]. Alone the MTRR 66A→G variant does not affect plasma homocysteine levels [92]. Homozygous individuals may have increased homocysteine concentrations when combined with low vitamin B_{12} concentrations [91, 93]. In addition, women with the 66GG genotype may have increased risk for having offspring with NTDs [94].

Homocystinuria can be caused by a severe deficiency in MTRR and results in hypomethioninemia, megaloblastic anaemia and low levels of methionine synthase [83]. Clinical symptoms include developmental delay, brain atrophy, nystagmus, hypotonia, seizures and abnormal electrophysiology [56, 83].

To examine the *in vivo* effects of MTRR deficiency a mouse model was developed and recently characterized [34]. A complete knockout of MTRR activity in mice results in embryonic lethality, therefore a gene-trap disruption in the *Mtrr* gene was used (*Mtrr*$^{Gt(pGT1Lxf)XG334Byg}$, hereafter abbreviated as *Mtrr*^{gt}. MTRR-deficient (*Mtrr*$^{gt/gt}$) mice have elevated plasma homocysteine and reduced plasma methionine levels. Adult male *Mtrr*$^{gt/gt}$ mice are significantly smaller in weight than wildtype. Additionally, our group has reported decreased embryonic length and weight, along with increased heart defects in offspring of *Mtrr*$^{gt/gt}$ mice [221]. MTRR is highly expressed in the developing neural tube and in adult mice *Mtrr* mRNA levels were significantly increased in *Mtrr*$^{gt/gt}$ mice [34]. In the present study we demonstrate that *Mtrr*$^{gt/gt}$ mice have short-term memory impairments along with reduced hippocampal volume and methylation as well as altered choline metabolism.

4.4 Results

Short-term memory disruption in *Mttr*$^{gt/gt}$ mice

Novel Object Recognition Test

During the test trial *Mttr*$^{gt/gt}$ spent more time (6 $\pm$ 2) with the familiar object when compared to *Mtrr*$^{+/+}$ (1.2 $\pm$ 0.5) mice ($p<0.001$). The discrimination index, a ratio of time spent with novel and familiar object during the testing trial showed global short-term memory impairment, in *Mttr*$^{gt/gt}$ animals (**Figure 4.1A**), *Mttr*$^{gt/gt}$ spent more time exploring the familiar object when compared to *Mtrr*$^{+/+}$ ($p<0.001$) *and Mtrr*$^{+/gt}$ ($p<0.001$) mice.

Y-maze Test

A difference was observed in the number of alternations made between genotype groups (**Figure 4.1B**, one-way ANOVA, p=0.05). There was no difference between genotype groups in other y-maze measurements.

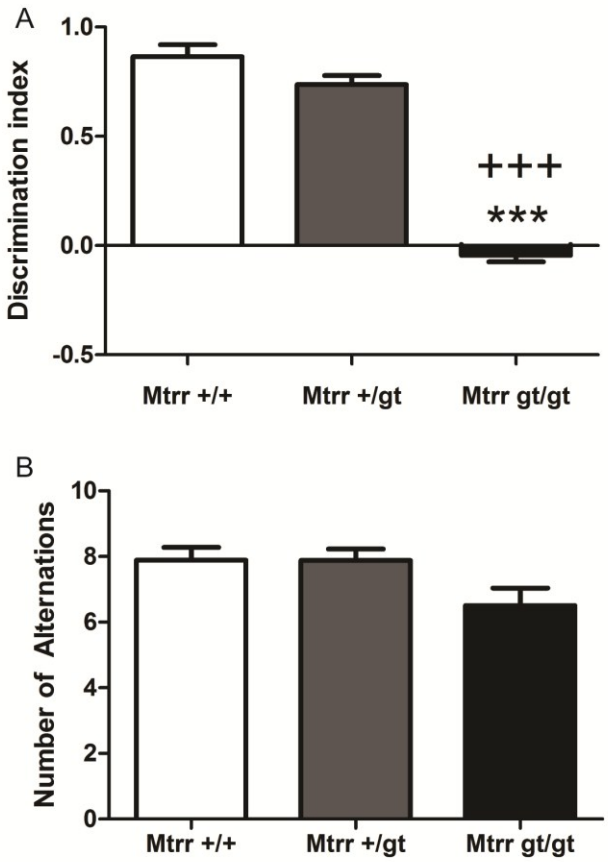

Figure 4.1 The effect of *Mtrr* genotype on short-term memory in 3-month-old male *Mtrr*$^{+/+}$, *Mtrr*$^{+gt-}$ and *Mtrr*$^{gt/gt}$ mice.

A, novel object recognition, discrimination index and B, y-maze, number of alternations. *Bars* represent mean ± standard error of the mean of eight to nine mice per group. *** p<0.01, Tukey's honestly significant difference post-hoc analysis, *Mtrr*$^{gt/gt}$ *vs.* *Mtrr*$^{+/+}$. +++p<0.001, Tukey's honestly significant difference post-hoc analysis, *Mtrr*$^{gt/gt}$ vs. *Mtrr*$^{+/gt}$. B, p<0.05, one-factor ANOVA, no significant differences in post-hoc analysis

Gait, motor function and affective behaviour changes in *Mtrr* $^{gt/gt}$ mice

Assessment of footprint patterns revealed that *Mtrr*$^{gt/gt}$ mice had altered stride length compared to *Mtrr*$^{+/gt}$ mice (**Figure 4.2A**; $p<0.05$). No change between genotype groups was observed in the movement score or percent error in the ladder beam walking task (data not shown).

Mttr$^{gt/gt}$ had decreased anxiety since they spent more time in the inside squares of the open field when compared to *Mtrr*$^{+/+}$ mice (**Figure 4.2B,** $p<0.05$). Furthermore *Mtrr*$^{gt/gt}$ and *Mtrr*$^{+/gt}$ had increased exploratory behaviour when compared to *Mtrr*$^{+/+}$ mice (**Figure 4.2C**, $p<0.05$).

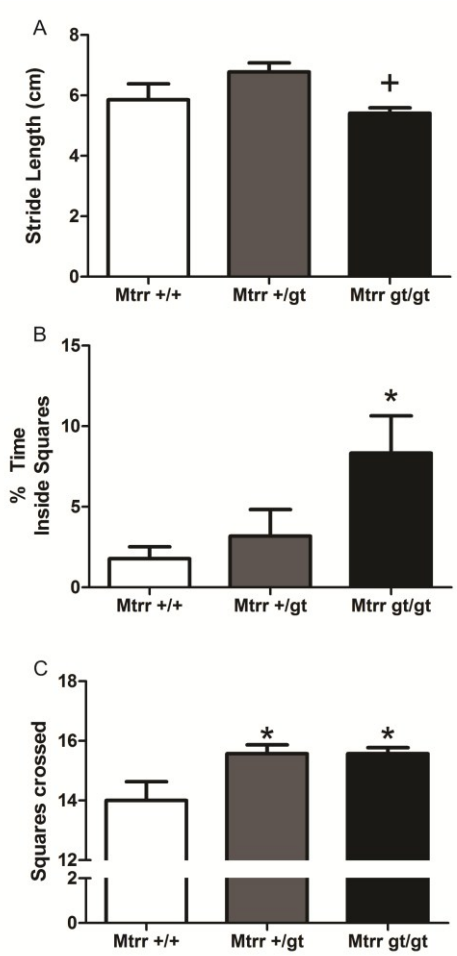

Figure 4. 2 The effect of *Mtrr* genotype on gait and affective behaviour levels on 3-month-old male *Mtrr*$^{+/+}$, *Mtrr*$^{+/gt}$ and *Mtrr* $^{gt/gt}$ mice.

A, footprint pattern stride length; B, open field, percent time spent inside squares; C, open field activity (number of squares crossed). *Bars* represent the mean $\pm$ standard error of the mean of five to nine mice per group. *$p<0.05$, Tukey's honestly significant difference post-hoc analysis, *Mtrr*$^{gt/gt}$ *or Mtrr*$^{+/gt}$ *vs. Mtrr*$^{+/+}$. +$p<0.05$, Tukey's honestly significant difference post-hoc analysis, *Mtrr*$^{gt/gt}$ *vs. Mtrr*$^{gt/+}$.

Plasma homocysteine in *Mtrr* $^{gt/gt}$ mice

Plasma total homocysteine levels were significantly elevated in *Mtrr*$^{gt/gt}$ (**Figure 4.3**) when compared to *Mtrr*$^{+/+}$ ($p<0.01$) and *Mtrr*$^{+/gt}$ ($p<0.05$) mice, as previously reported in [34].

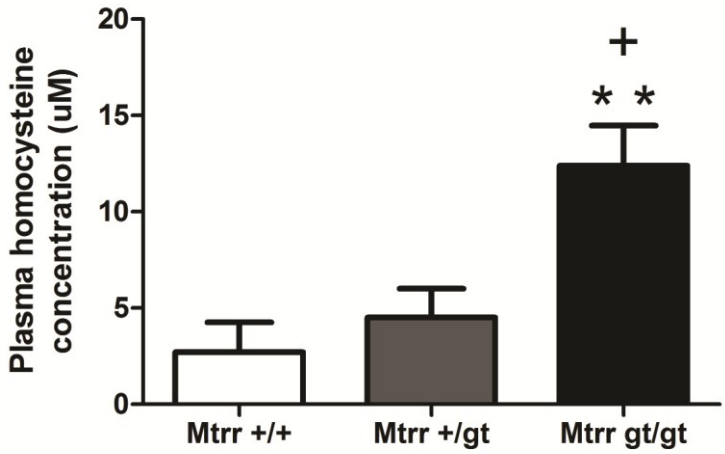

Figure 4. 3 The effect of *Mtrr* genotype on plasma homocysteine in 3-month old male *Mtrr*$^{+/+}$, *Mtrr*$^{+/gt}$ and *Mtrr* $^{gt/gt}$ mice.

Bars represent the mean ± standard error of the mean of six mice per group. **$p<0.01$, Tukey's honestly significant difference post-hoc analysis, *Mtrr*$^{gt/gt}$ *vs.* *Mtrr*$^{+/+}$. +$p<0.05$, Tukey's honestly significant difference post-hoc analysis, *Mtrr* $^{gt/gt}$ vs. *Mtrr* $^{+/gt}$.

Hippocampal morphology, MRI-defined volume and global DNA methylation

No changes in thickness of CA1 CA3 and dentate gyrus were observed between groups. *Mttr*$^{gt/gt}$ and *Mttr*$^{+/gt}$ mice had smaller hippocampal volume when compared to *Mttr*$^{+/+}$ mice (**Figure 4.4A**, $p<0.05$). No changes in whole brain volume were observed between genotype groups. Global hypomethylation was

observed in *Mtrr*$^{gt/gt}$ when compared to *Mtrr*$^{+/+}$ and *Mtrr*$^{+/gt}$ mice (**Figure 4.4B**, $p<0.01$).

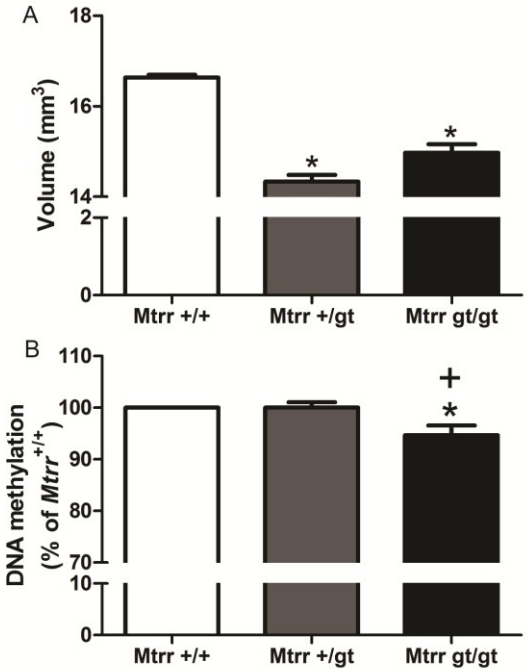

Figure 4. 4 The effect of *Mtrr* genotype on hippocampal A, MRI volume and B, global DNA methylation of 3-month-old male *Mtrr*$^{+/+}$, *Mtrr*$^{+/gt}$ and *Mtrr*$^{gt/gt}$ mice.

Bars represent the mean ± standard error of the mean of two to three mice per group for MRI volume and five to six mice per group for methylation. *$p<0.05$, Tukey's honestly significant difference post-hoc analysis, *Mtrr*$^{gt/gt}$ or *Mtrr*$^{+/gt}$ *vs.* *Mtrr*$^{+/+}$. +$p<0.05$, Tukey's honestly significant difference post-hoc analysis, *Mtrr*$^{gt/gt}$ *vs.* *Mtrr*$^{gt/+}$.

Choline metabolism in MTRR-deficient animals

In the hippocampus there was a genotype difference in choline levels (**Table 4.1**, ANOVA, $p=0.05$). A trend for genotype difference for levels of betaine

and acetylcholine were observed (ANOVA, p=0.06). No changes were observed in other choline metabolites were observed.

Table 4. 1 Concentration of hippocampal and plasma choline metabolites in *Mtrr*$^{+/+}$, *Mtrr* $^{+/gt}$ and *Mtrr*$^{gt/gt}$mice[1]

	Hippocampus				Plasma			
Genotype	*Mtrr* +/+	*Mtrr* +/*gt*	*Mtrr gt* /gt	*p* value [2]	*Mtrr* +/+	*Mtrr* +/*gt*	*Mtrr gt* /gt	*p* value [2]
Metabolite (nmol/g)								
Choline	87.5 ± 6	68 ± 6	70 ± 4	p=0.05	17 ± 3	21 ± 2	20 ± 2	NS
Betaine	14 ±1	12 ± 1	9 ± 1	p=0.06	57 ± 4	63 ± 4	80 ± 9	p<0.05
Acetylcholine	4 ± 0.5	3.3 ± 0.4	2.7 ± 0.1	p=0.06	—	—	—	—
Phosphatidylcholine	22023 ± 380	22151 ± 387	21626 ± 680	NS	2002 ± 107	1591 ± 151	1731 ± 162	NS
Glycerophosphocholine	817 ± 28	756 ± 83	816 ± 32	NS	17 ± 1	20 ± 2	19 ± 3	NS
Phosphocholine	533 ± 14	549 ± 27	553 ± 7	NS	3 ± 0.1	4 ± 0.3	3 ± 0.3	NS
Sphingomyelin	3209 ± 77	3129 ± 64	3176 ± 85	NS	48 ± 5	47 ± 1	48 ± 3	NS

[1] All values are means ± standard error of 6 to 7 mice per group.

[2] *p* values were derived from one-factor ANOVA comparisons between genotype groups.

Since *Bhmt* is not expressed in the brain, choline cannot be used as a methyl donor to remethylate homocysteine to methionine. Our findings suggest that the oxidation of choline to betaine by CHDH is reduced in the hippocampus of *Mtrr*$^{gt/gt}$ mice this may be occurring so that more choline is available for Ach and phosphatidycholine biosynthesis.

To further examine the influence of *Mtrr* deficiency on Ach biosynthesis, we measured immunoreactive protein levels of ChAT, which catalyzes synthesis of acetylcholine. In the hippocampus (**Figure 4.5**), immunoreactive protein levels of ChAT were significantly increased in *Mtrr*$^{gt/gt}$ animals when compared to *Mtrr*$^{+/+}$ ($p<0.05$) and *Mtrr*$^{+/gt}$ ($p<0.01$). These findings suggest that up-regulation of acetylcholine synthesis may occur in gene trapped animals to replenish this critical neurotransmitter.

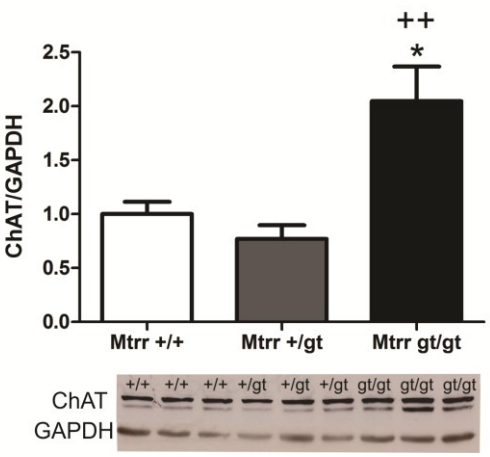

Figure 4.5 The effect of *Mtrr* genotype on hippocampal choline acetyltransferase (ChAT) protein levels of 3-month-old male $Mtrr^{+/+}$, $Mtrr^{+/gt}$ and $Mtrr^{gt/gt}$ mice.

Bars represent the mean ± standard error of the mean of five mice per group. *p<0.05, Tukey's honestly significant difference post-hoc analysis, $Mtrr^{gt/gt}$ *vs.* $Mtrr^{+/+}$ or $Mtrr^{+/gt}$ *vs.* $Mtrr^{+/+}$. ++p<0.01, Tukey's honestly significant difference post-hoc analysis, $Mtrr^{gt/gt}$ *vs.* $Mtrr^{gt/+}$. The panel below the graph depicts a representative Western blot.

In the plasma there was no change in choline levels, however there was a genotype difference in plasma betaine levels (**Table 4.1**, ANOVA, p<0.05). Post-hoc analysis revealed that $Mtrr^{gt/gt}$ had elevated levels of plasma betaine when compared to $Mtrr^{+/+}$ mice (p<0.05). Analysis of choline metabolism in the liver showed reduced mRNA expression of *Bhmt* in the liver of $Mtrr^{gt/gt}$ when compared to $Mtrr^{+/+}$ animals (**Figure 4.6A**, p<0.05), as well as expression of *Chdh* when compared to $Mtrr^{+/gt}$ animals (**Figure 4.6B**, p<0.05).

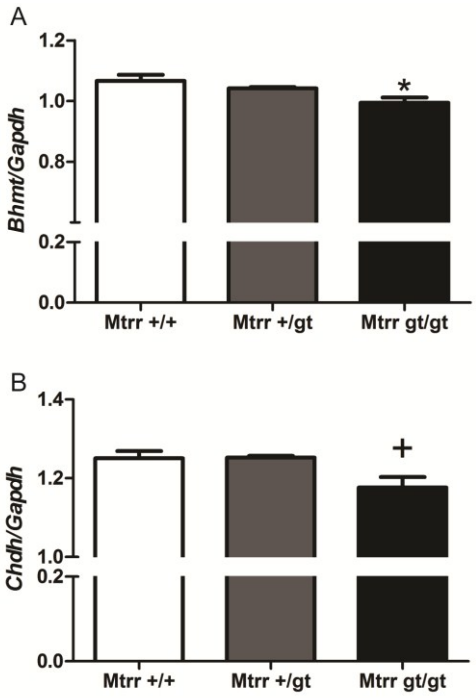

Figure 4. 6 The effect of *Mtrr* genotype on liver mRNA expression of A, betaine homocysteine methyltransferase (*Bhmt*) and B, choline dehydrogenase (*Chdh*) in 3-month old $Mtrr^{+/+}$, $Mtrr^{+/gt}$ and $Mtrr^{gt/gt}$ mice.

Bars represent the mean ± standard error of the mean of four mice per group. *p<0.05, Tukey's honestly significant difference post-hoc analysis, $Mtrr^{gt/gt}$ *vs.* $Mtrr^{+/+}$. +p<0.05, Tukey's honestly significant difference post-hoc analysis, $Mtrr^{gt/gt}$ *vs.* $Mtrr^{gt/+}$.

Morphology and neurobiochemical changes in cerebellum

Changes in cerebellar morphology and function have been described in severe MTHFR-deficient mice [35, 150, 222], we therefore examined cerebellar function and morphology in MTRR-deficient mice in the present study. There was no difference between genotype groups in cerebellar volume or morphology. Cerebellar global hypomethylation was observed in $Mtrr^{gt/gt}$ mice when compared to $Mtrr^{+/+}$ and $Mtrr^{+/gt}$ mice (**Figure 4.7A**). A decrease in choline levels in the cerebellum was observed in $Mtrr^{+/gt}$ mice when compared to $Mtrr^{+/+}$ mice (**Figure**

4.7B). Other choline metabolites including Ach levels were not affected by genotype in the cerebellum. However, choline acetyltransferase (ChAT) levels were elevated in *Mtrr*$^{gt/gt}$ mice compared to *Mtrr*$^{+/+}$ mice (**Figure 3.7C**).

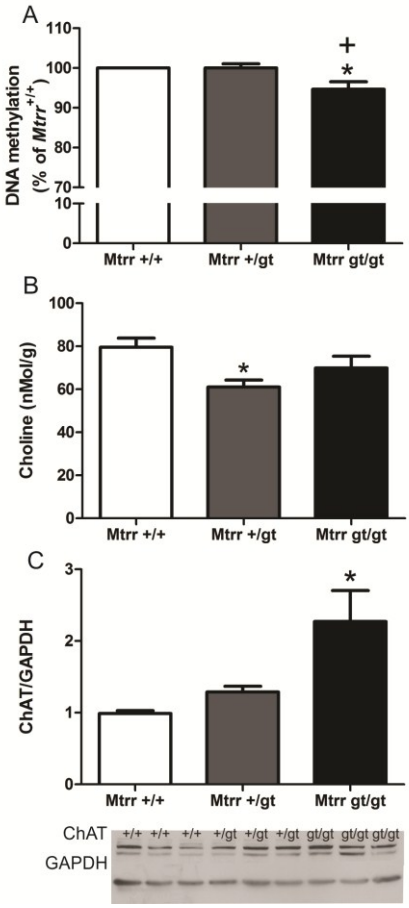

Figure 4.7 The effect of *Mtrr* genotype on cerebellum biochemistry.

A global DNA methylation; B, choline metabolite levels and C, choline acetyltransferase (ChAT) protein levels of 3-month-old male *Mtrr*$^{+/+}$, *Mtrr*$^{+/gt}$ and *Mtrr* $^{gt/gt}$ mice. *Bars* represent the mean $\pm$ standard error of the mean of five to nine mice per group. *$p<0.05$, Tukey's honestly significant difference post-hoc analysis, *Mtrr*$^{gt/gt}$ or *Mtrr*$^{+/gt}$ *vs.* *Mtrr*$^{+/+}$. +$p<0.05$, Tukey's honestly significant difference post-hoc analysis, *Mtrr*$^{gt/gt}$ *vs.* *Mtrr*$^{gt/+}$. C, the panel below the graph depicts a representative Western blot.

4.5 Discussion

We confirmed previously reported mildly elevated levels of plasma homocysteine in *Mtrr*$^{gt/gt}$ mice [34]. To avoid any gender-related difference in memory and motor function in the present study, we used only male mice [154]. We identified short-term memory impairments, gait abnormalities and changes in affective behaviour in male *Mtrr*$^{gt/gt}$ mice with mild-hyperhomocysteinmia. Impairments in short-term memory may be a result of changes we identified in the hippocampus including reduced volume, global DNA hypomethylation and altered choline metabolism. The changes in motor function are less severe but may also be a result of altered choline metabolism in the cerebellum. We identified increased levels of betaine in plasma and decreased expression of *Chdh and Bhmt* in liver of *Mtrr*$^{gt/gt}$ mice, which maybe a result of redistribution of choline during MTRR deficiency [223].

Hippocampus and mild hyperhomocysteinmia in an *in vivo* animal model

It is well established that the hippocampus is involved in cognitive function, specifically learning and memory [102]. Epidemiological research has suggested that elevated plasma homocysteine levels negatively influence cognitive function [224-226] and are associated with hippocampal atrophy [203, 227, 228]. Our group has shown that a genetic disruption in folate metabolism (e.g. severe MTHFR deficiency) results in morphological changes in the hippocampus of mice [152, 222]. In the present study we show adult mice with mild-hyperhomocysteinmia have impaired short-term memory along with decreased hippocampal volume. A study by [229] reported that hippocampal cells are more susceptible to elevated levels of homocysteine than cortical cells. The possible mechanisms through which

homocysteine exerts its neurotoxic effects on hippocampal neurons include repeated excitation via the NMDA receptor [138, 139], increased oxidative stress [137] and apoptosis [222, 230].

Hypomethylation in the hippocampus and cerebellum was also observed in *Mtrr*$^{gt/gt}$ mice. A decrease in DNA methylation can have an impact on gene expression and, consequently, on disease development [231]. The hippocampus is one of the only structures in the brain that undergoes neurogenesis during adulthood [104]. We suggest that decreased levels of methylation and/or elevated levels of homocysteine may affect hippocampal neurogenesis and therefore impair hippocampal function.

Disrupted choline metabolism in *Mtrr*$^{gt/gt}$ mice

In the present study we observed that choline metabolite levels were more affected in hippocampus versus the cerebellum. In the hippocampus we observed reduced levels of choline, betaine and Ach in *Mtrr*$^{gt/gt}$ mice, whereas in the cerebellum we observed decreased levels of choline in *Mtrr* $^{+/gt}$ mice, no changes in *Mtrr*$^{gt/gt}$ mice were observed. Choline and its metabolite betaine do not serve as alternative methyl donors in the brain [64]. However, choline in the brain is important for cell membrane integrity and generation of the neurotransmitter, acetylcholine [63]. Ach plays a central role in learning and memory [206], we investigated protein levels of ChAT, an enzyme involved in the production of Ach and report elevated levels of ChAT in both the hippocampus and cerebellum. Increased levels of ChAT have been suggested to compensate for decreased levels of Ach [207].

In the plasma of *Mtrr*$^{gt/gt}$ mice we identified elevated levels of betaine along with decreased expression of *Chdh* and *Bhmt* in the liver. Choline and betaine, with the help of BHMT can be used as an alternative methyl donor in the liver and kidney to maintain normal levels of homocysteine [38], choline pools in theses tissue may become depleted. The increased levels of betaine in the plasma and decreased levels of *Chdh* and *Bhmt* in the liver observed in *Mtrr*$^{gt/gt}$ may be a result of redistribution of choline metabolites during MTRR deficiency [223]. It is important to note that the absolute value changes in *Chdh* and *Bhmt* mRNA expression between groups were small and these small changes may not be biologically significant.

Epidemiological research has described that lowering homocysteine by B-vitamin administration of individuals is associated with reducing brain atrophy [225] as well as improving cognitive function [124, 232]. *In vitro* work has shown that folic acid supplementation during folic acid deficiency results in decreased levels of apoptosis [130]. In mice, maternal betaine supplementation during pregnancy ameliorated some of cerebellar defects, but not all, associated with a severe MTHFR deficiency in offspring. The data from the present study suggest that dietary supplementation with choline in the case of MTRR deficiency to ameliorate the negative effects in brain tissuemay provide some benefit to affected individuals.

Homocystinuria induced via MTHFR or MTRR

Homocystinuria can be a result of a deficiency in either MTHFR or MTRR [83]. *In vivo* investigation of severe MTHFR deficiency, a mouse model for homocystinuria, revealed significantly elevated plasma homocysteine levels, along

with changes in affective behaviours and impairments in motor and cognitive function [96, 222]. In addition, morphological and biochemical changes were described in the cerebellum and hippocampus that may lead to the behavioural phenotype. In contrast, *Mtrr*$^{gt/gt}$ mice have mildly elevated plasma homocysteine levels reported in this study and previously in [34, 96]. Behaviourally, we report impairments in short-term memory, changes in affective behaviours and gait, in addition to hippocampal and cerebellar biochemical changes. The behavioural, morphological and biochemical phenotypes observed in *Mtrr*$^{gt/gt}$ are not as severe as those observed in *Mthr*$^{-/-}$ mice. This may be a result of the significantly elevated levels of plasma homocysteine in MTHFR-deficient mice. *Mthfr* knockout mice have decreased levels of SAM along with DNA hypomethylation in brain tissue [35, 222]. These decreases could potentially also result in reduced neurotransmitter synthesis and in changes in lipid metabolism. Changes in SAM levels in MTRR-deficient mice have not yet been reported; however, in the present study we observed DNA hypomethylation in the hippocampus, In summary we have observed impairments in short-term memory, changes in gait function and affective behaviours in a mouse model for mild-hyperhomocysteinemia. Choline metabolism was significantly altered in the hippocampus, plasma and liver of *Mtrr*$^{gt/gt}$ mice. The short-term memory impairments may be a result of reduced hippocampal volume, hypomethylation or decreased levels of acetylcholine in the hippocampus. Mild hyperhomocysteinmia has been linked to the development of cognitive impairment associated with neurodegeneration [226, 229, 233, 234]. To understand the mechanism of action through which mild-hyperhomocysteinmia affects cognitive function and neurodegeneration *in vivo* it may be worthwhile to

investigate the cognitive function and biochemical changes in aged MTRR-deficient mice.

4.6 Acknowledgements

We thank Barry J. Bedell, Marie A. Caudill, Eve-Marie Charbonneau , Liyuan Deng, Marilyn Grand'Maison, Olga Malysheva, Xiao-ling Wang and Qing Wu, and for technical assistance. This work was supported by the Canadian Institutes of Health Research. NMJ was a recipient of the Charles Banting and Frederick Best Canadian Institutes of Health Research Graduate Scholarship.

CONNECTING TEXT – Chapters IV-V

MTRR is essential in the reactivation of the MTR. Patients with a severe MTRR deficiency have neurological symptoms [85]. In chapter IV we confirmed mildly elevated levels of plasma homocysteine in gene-trapped (*Mtrr*$^{gt/gt}$) mice. We identified short-term memory impairments, along with changes in gait and affective behaviours and observed reduced volume and global DNA methylation in the hippocampus in adult *Mtrr*$^{gt/gt}$ mice. Interestingly, altered choline metabolites were observed in the hippocampus, cerebellum and plasma of these mice. In the liver of *Mtrr*$^{gt/gt}$ mice we observed decreased mRNA expression of *Chdh* and *Bhmt.* These data are interesting because they show that mildly elevated levels of plasma homocysteine may result in short-term memory impairments that are accompanied with biochemical changes in the hippocampus.

The first two chapters of this thesis investigated the effects of a MTHFR and MTRR genetic deficiency on brain function. The next two chapters will investigate the role of maternal contributions of MTHFR, folic acid and choline in brain function and structure of wild type offspring. A maternal MTHFR deficiency results in offspring with embryonic delays, decreased embryonic crown-rump length and weight and congenital heart defects [163, 164]. Chapter V will investigate the effect of maternal MTHFR deficiency on brain function and structure of 3-week-old *Mthfr*$^{+/+}$ male mice.

CHAPTER V: Maternal MTHFR deficiency results in altered motor function, impaired short-term memory and increased apoptosis in male *Mthfr*$^{+/+}$ offspring

5.1 Abstract

Elevated levels of maternal plasma homocysteine have been linked to negative health outcomes in offspring. Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in the metabolism of homocysteine. Maternal deficiencies in MTHFR have been reported to result in embryonic delays, decreased embryonic crown-rump length and weight and increased resorption rate, as well as congenital heart defects in offspring. The purpose of this study was to investigate the role of maternal MTHFR deficiency on brain function of 3-week-old male *Mthfr*$^{+/+}$ offspring. *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ females were placed on a control diet for 6 weeks and then mated with *Mthfr*$^{+/-}$ males. We assessed short-term memory, skilled motor function and affective behavior in *Mthfr*$^{+/+}$ offspring 3 weeks of age. Brain tissue was evaluated for cerebellar and hippocampal morphological changes, global methylation, apoptosis, gene expression of some enzymes involved in folate metabolism and proteins levels of choline acetyltransferase (ChAT). We observed that *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mother had impairments in short-term memory. These mice also had changes in gait function, as well as impaired skilled motor function. *Mthfr*$^{+/-}$ mothers had elevated levels of plasma homocysteine when compared to *Mthfr*$^{+/+}$ mothers, no change in plasma homocysteine was observed in offspring according to maternal genotype. There were no changes in cerebellar and hippocampal morphology, global methylation, gene expression or protein levels in the brain of offspring from *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mothers. Increased apoptosis was observed in both the cerebellum and hippocampus of *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mothers. Our results suggest that a deficiency in maternal MTHFR results in impairments in

short-term memory and motor function and that these behavioural changes may be modulated by increased maternal plasma homocysteine levels and apoptosis in offspring cerebellum and hippocampus.

5.2 Introduction

Elevated maternal or offspring plasma homocysteine levels have been linked to increased risk of neural tube defects (NTDs) [141] and to negative effects on fetal brain development affecting normal function later in life [235]. In the developing brain elevated levels of homocysteine have been shown to increase oxidative stress [230] and apoptosis [222].

Methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) is a key enzyme in the metabolism of folate and homocysteine, it catalyses the synthesis of 5-methyltetrahydrofolate (5-methylTHF), the main circulatory form of folate. 5-methylTHF is used for remethylation of homocysteine to methionine and *S*-adenosylmethionine (SAM), a universal methyl donor [30].

A polymorphism in MTHFR (677C→T) converting an alanine to valine residue results in mild MTHFR deficiency [51]. Homozygous individuals for this polymorphism have reduced enzyme activity and lowered plasma folate, along with elevated levels of plasma homocysteine. Homozygosity is present in 5-20% of North American and European populations [236]. Furthermore, maternal [237] or offspring [141] deficiencies in MTHFR have been associated with increased risk for development of neural tube defects (NTDs). The risk of NTDs increases significantly when both the mother and offspring are homozygous [237]. Furthermore, a maternal MTHFR deficiency has also been associated with increased risk for thromboembolic events [230]. Females with the 677 *MTHFR*

variant require folate supplementation during pregnancy [238, 239]. In 1998, the US and Canada implemented mandatory folate fortification, this has resulted in decrease prevalence of NTDs [167].

To examine the *in vivo* effects of MTHFR deficiency, a mouse model for mild MTHFR deficiency was developed in our laboratory [35]. *Mthfr*$^{+/-}$ mothers on a control diet (2 mg/kg folic acid) have increased resorption rates and give rise to offspring with embryonic delays [163, 164], decreased embryonic crown-rump length and weight [164] , along with increased congenital heart defects [163]. A study by [230] reported offspring from hyperhomocysteinemic mothers have increased levels of oxidative stress in brain tissue that may lead to apoptosis. The purpose of this study was to investigate the effect of maternal MTHFR deficiency on brain function of *Mthfr*$^{+/+}$ offspring. We report wild type 3-week-old offspring from *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mothers have changes in motor function and short-term memory along with increased apoptosis in the cerebellum and hippocampus.

5.3 Results

Behavioural Function

Mthfr$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mother had decreased print length (**Figure 5.1A**, $p<0.01$) when compared to offspring from *Mthfr*$^{+/+}$ mothers. *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mothers made more errors on the ladder beam walking task when compared to offspring from *Mthr*$^{+/+}$ mothers (**Figure 5.1B**, $p<0.05$). No change in movement score was observed between groups.

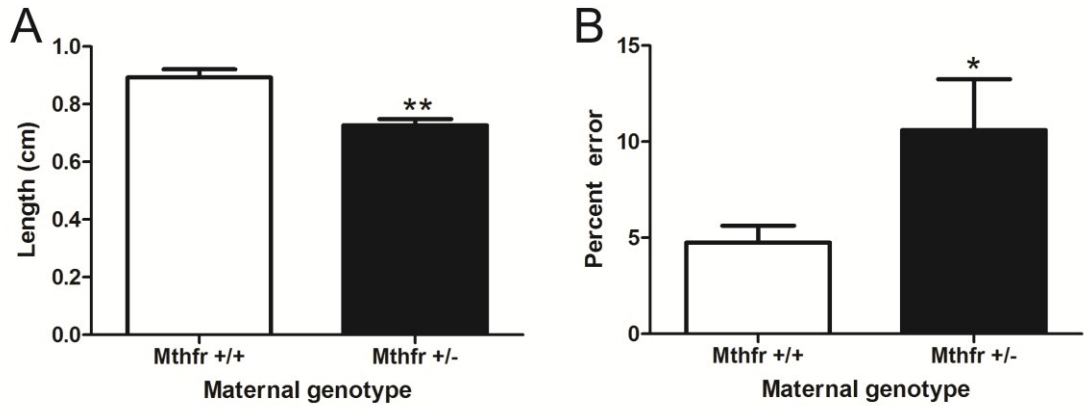

Figure 5.1 Effect of maternal *Mthfr* on gait (print length; footprint pattern) and skilled motor function (% error; ladder beam walking task) in 3-week-old *Mthfr*$^{+/+}$ offspring.

A, print length; B, percent error. Bars represent mean ± standard error of 8 to 10 mice per group. *p<0.05, **p<0.01, un-paired t-test, maternal *Mthfr*$^{+/-}$ vs. *Mthfr*$^{+/+}$.

During the test trial of the novel object recognition task there was no difference between groups for the amount of time spent with novel versus familiar object. However, the discrimination index, a ratio of time spent with familiar and novel object during the test phase showed that *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mothers spent more time with the familiar object during the test period compared to offspring from *Mthfr*$^{+/+}$ mothers (**Figure 5.2A**, $p<0.05$). There was no difference in number of alternations made in the y-maze between groups (**Figure 5.2B**, $p=0.10$).

No changes in affective behaviour were observed between groups.

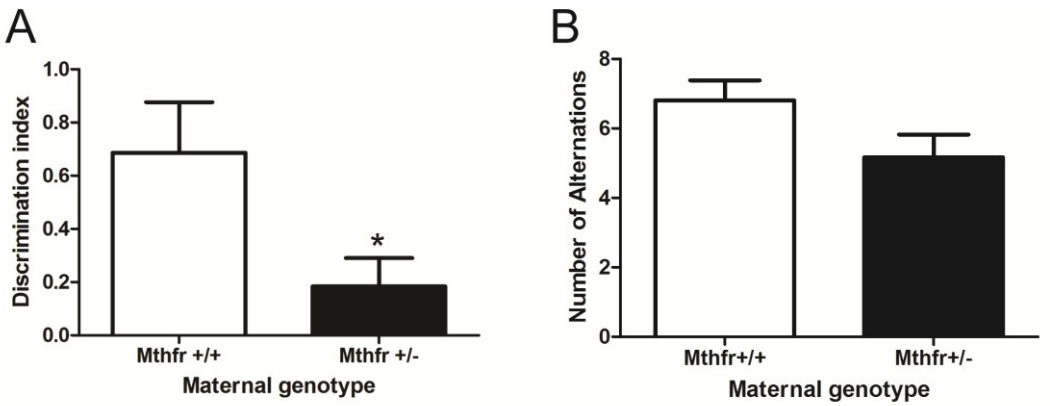

Figure 5.2 Effect of maternal *Mthfr* on short-term memory in 3-week-old *Mthfr*$^{+/+}$ offspring.

A, discrimination index novel object recognition task. B, number of alternations, y-maze. Bars represent mean ± standard error of 8 to 10 mice per group. *p<0.05, un-paired t-test, maternal *Mthfr*$^{+/-}$ vs. *Mthfr*$^{+/+}$. B, un-paired t-test, p=0.10.

Body and brain weight, plasma homocysteine, cerebellar and hippocampal morphology, global DNA methylation

There was no difference in body and brain weight between groups. *Mthfr*$^{+/-}$ mothers had elevated plasma homocysteine concentrations when compared to *Mthfr*$^{+/+}$ (**Figure 5.3A**, p<0.01). No difference in plasma homocysteine concentrations in *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mothers (**Figure5.3B**). No changes in cerebellar and hippocampal morphology and global DNA methylation were observed between groups.

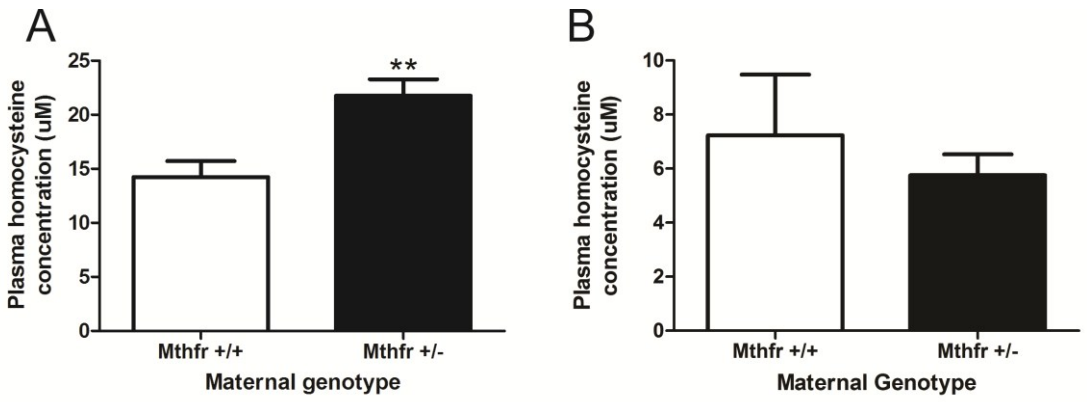

Figure 5.3 Plasma homocysteine concentrations.

A, maternal plasma homocysteine of 3-month-old *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ pregnant females; B, offspring plasma homocysteine of 3-week old *Mthfr*$^{+/+}$ pups from *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ females. Bars represent mean $\pm$ standard error of 10 to 12 mice per group. **p<0.01, un-paired t-test, maternal *Mthfr*$^{+/-}$ vs. *Mthfr*$^{+/+}$.

Cerebellar and hippocampal Apoptosis

Increased apoptosis was observed in *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mothers in the cerebellum (**Figure 4.4A**, $p<0.05$) and hippocampus (**Figure 4.4B**, $p<0.05$). More apoptotic cells were observed in the dentate gryrus of the hippocampus than the CA1 CA3 region.

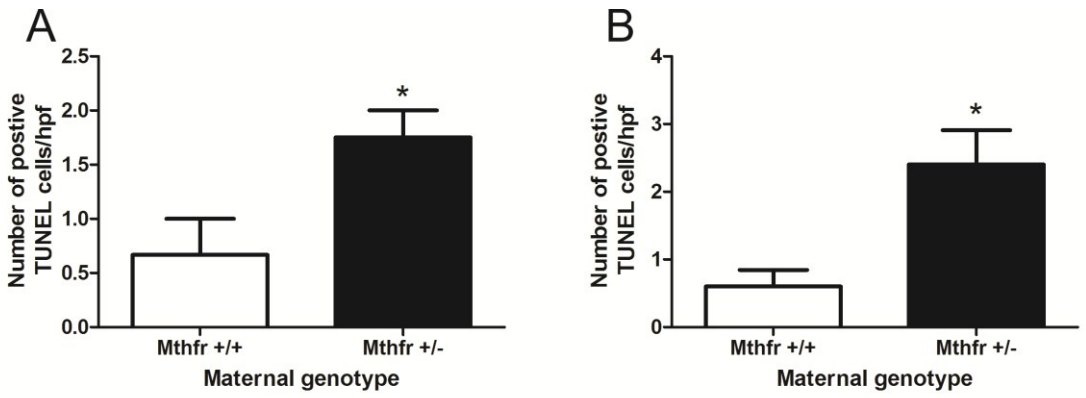

Figure 5.4 Effect of maternal *Mthfr* on apoptosis in A, cerebellum and B, hippocampus in 3-week-old *Mthfr*$^{+/+}$ offspring.

Bars represent mean ± standard error of 6 to 8 mice per group. *p<0.05, un-paired t-test, maternal *Mthfr*$^{+/-}$ vs. *Mthfr*$^{+/+}$.

Gene expression and protein levels

In the hippocampus, no change in mRNA expression of GR, MTR and MTRR was observed between groups. Data not shown.

Mthfr$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mother had a trend for increased protein levels of GR in hippocampus when compared to offspring from *Mthfr*$^{+/+}$ mothers (p=0.06). Data not shown.

No change in protein levels of ChAT were observed in the cerebellum and hippocampus between maternal genotype groups. Data not shown.

5.4 Discussion

In the present study we confirmed that *Mthfr*$^{+/-}$ mothers had elevated levels of plasma homocysteine as previously reported [164]. Sex differences between males and females have previously been reported, therefore we limited our study to male offspring in order to avoid this confounder effect [154]. We identified impairments in motor and short-term memory on the novel object recognition task,

but not on the y-maze, in male 3-week-old wild-type offspring from *Mthfr*$^{+/-}$ mothers. Increased apoptosis was observed in the cerebellum and hippocampus in *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mothers.

Maternal MTHFR deficiency

One study reported an increased risk for neurocognitive deficits in babies from mothers homozygous for the 677 *MTHFR* variant [162]. Furthermore, neurodevelopment of babies can be negatively affected when mothers are deficient in B-vitamins including folate and vitamin B_{12} [235, 240]. A study by [241] reported no association between maternal folate nutritional status during the second half of pregnancy and motor and psychomotor development in offspring, however, these data may not be representative of the population since samples were collected at one hospital . Our group has shown that a maternal MTHFR deficiency results in negative outcomes in offspring using a mouse model [163, 164]. The present study adds changes in brain function to the list of negative outcomes of a maternal MTHFR deficiency observed in mice offspring.

Effect of elevated maternal plasma homocysteine levels on offspring brain development and function

An epidemiological study did not report an association between maternal homocysteine levels to birth weight of babies [242] and in the present study we observed similar findings in mice. A study by [230] showed that hyperhomocysteinemic female rat dams gave rise to offspring with increased levels of oxidative stress and apoptosis in brain. In the present study we observed elevated levels of apoptosis in the cerebellum and hippocampus of offspring from *Mthfr*$^{+/-}$ mothers. Indeed *Mthfr*$^{+/-}$ mothers had elevated levels of plasma

homocysteine. Homocysteine is responsible for the activation of pro-apoptotic molecules including p53 and Bax [136, 243, 244]. Furthermore, homocysteine has been shown to accumulate in the cerebellum and hippocampus of rat offspring from hyperhomocysteinemic mothers [243]. Maternal hyperhomocysteinemia has been reported to also lead to elevated levels of homocyteine in offspring [245], however, in the present study we did not make the same observation. This may be due to the fact that all offspring were *Mthfr*$^{+/+}$.

Evaluating maternal choline metabolism

We evaluated choline acetyltransferase (ChAT) protein levels in cerebellum and hippocampus of wild-type offspring, since we have previously reported that a MTHFR deficiency results in changes in this protein [222], and observed no difference between groups. It might be warranted to evaluate choline metabolism of the *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ mothers, since folate and choline metabolism are tightly linked [38]. Additionally, previous work by [172] has shown that pregnant BALB/c *Mthfr*$^{+/-}$ mothers maintained on a control diet have elevated levels of choline in plasma. Liver choline metabolite analysis in pregnant *Mthfr*$^{+/+}$ and *Mthfr*$^{+/-}$ BALB/c mice revealed no changes between groups [246].

In summary this study has identified changes in motor function and impairments in short-term memory of 3-week-old offspring as a result of maternal MTHFR deficiency. Elevated levels of maternal plasma homocysteine and/or increased apoptosis in brain tissue of offspring may lead to behavioural changes reported in *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mothers. Further studies are required to determine whether long-term memory may be affected by a maternal MTHFR

deficiency using the standard Morris watermaze along with other potential mechanisms.

5.7 Acknowledgements

We thank Liyuan Deng, Leonie Mikael, Qing Wu, and Eve-Marie Charbonneau for technical assistance. NMJ was a recipient of the Charles Banting and Frederick Best Canadian Institutes of Health Research Graduate Scholarship.

CONNECTING TEXT – Chapters V-VI

Mthfr$^{+/-}$ mice present a clinical phenotype that mimic the polymorphism at base pair 677C→T in humans. This polymorphism is present in 5-20% of North American and European populations [87]. In chapter 5 we observed that *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mothers have impairments in short-term memory and skilled motor function. These mice also have increased levels of apoptosis in the hippocampus and cerebellum.The results of this study are interesting, but require further confirmation. During pregnancy adequate amounts of folate and choline are required for closures of the neural tube in offspring [68, 115]. The long term effects of folate or choline deficiency are not fully understood on brain function. In Chapter VI we investigate the impact of maternal folic acid or choline deficiency on brain function and structure in 3-week-old wild type offspring.

CHAPTER VI: Deficiencies of maternal folic acid or choline result in changes of brain function in 3-week-old *Mthfr*$^{+/+}$ offspring

6.1 Abstract

Maternal demands for folic acid and choline are increased during pregnancy. Previous work has shown that maternal folic acid or choline deficiencies result in negative outcomes in offspring including increased resorption rate and delayed embryos. The purpose of this study was to investigate the impact of FADD or ChDD during pregnancy on brain function of offspring. *Mthfr*$^{+/+}$ females were placed on FADD, ChDD and CD for 6 weeks and then mated with *Mthfr*$^{+/-}$ males. We assessed short-term memory, skilled motor function and affective behavior in 3-week-old *Mthfr*$^{+/+}$ offspring. Plasma homocysteine was measured in mothers and offspring. Brain tissue was evaluated for cerebellar and hippocampal morphological changes, apoptosis, choline metabolites and protein levels of choline acetyltransferase (ChAT). We observed impairments in short-term memory in *Mthfr*$^{+/+}$ offspring from FADD or ChDD mothers. In *Mthfr*$^{+/+}$ offspring from ChDD mothers, we noted impaired motor function whereas offspring from FADD mothers showed increased activity and decreased anxiety. *Mthfr*$^{+/+}$ offspring from FADD mothers had a trend for increased plasma homocysteine levels. Increased apoptosis, changes in choline metabolites and increased choline acetyltransferase protein levels were observed in both the cerebellum and hippocampus of *Mthfr*$^{+/+}$ offspring from FADD and ChDD mothers. Our results suggest that a deficiency in maternal folic acid or choline results in behavioural changes in *Mthfr*$^{+/+}$ offspring which may be modulated by increased plasma homocysteine levels, apoptosis or altered choline metabolites in the cerebellum and hippocampus.

6.2 Introduction

The demand for folate is increased during pregnancy to accommodate development of embryo and fetus, as well as growth of maternal tissue [247, 248]. Folic acid is required for cellular proliferation including, as well as maintaining normal levels of homocysteine and facilitating methylation reactions [16, 22, 248]. In order for proper brain and spinal cord development ffolate is required for neural tube closure in the developing fetus, deficiencies result in NTDs in offspring [24]. In 1998, the US and Canada fortified cereals and grains with folic acid to help reduce the incidence of NTDs [68]. Recently a follow-up study has shown that fortification led to decreased number of neural tube defects [167].

Choline is an essential nutrient and can also be synthesized *de novo* [59]. The requirement of choline is increased during pregnancy [61]. For brain development during early pregnancy it is required for normal closure of the neural tube [115, 249] and then later in pregnancy for synthesis of the neurotransmitter acetylcholine, neurogenesis and myelination [250]. Throughout pregnancy choline is required to maintain normal levels of plasma homocysteine [61, 250]. Maternal stores can be depleted during pregnancy and lactation since choline is transported against a concentration gradient to the fetus and breast milk in order for the developing fetus and neonate to have an adequate supply of the nutrient [251]. Interestingly, both folic acid and choline are required later in pregnancy for the development of the hippocampus [115].

In mice, maternal FADD has been shown to result in an increase in resorption rate, delayed embryos and congenital heart defects in offspring [163]. MTHFR, a key enzyme in the metabolism of folate and homocysteine, catalyzes

the synthesis of 5-methyltetrahydrofolate (5-methylTHF), the main circulatory form of folate. Maternal deficiencies in MTHFR and FADD result in decreased embryonic weight, crown-rump length and placenta weight and area, as well as decreased number of viable embryos [164, 252]. Increased levels of maternal plasma homocysteine have also been reported [164]. When maternal ChDD is combined with maternal MTHFR deficiency there are decreased choline metabolites in plasma of pregnant females, along when increased levels of plasma homocysteine levels [172, 176]. The outcomes of maternal dietary FADD and ChDD, along with MTHFR deficiencies may be a result of decreased apolipoprotein AI (ApoA-I) [164, 176]. In addition, maternal FADD and ChDD have reported to led to altered inflammation in pregnant mothers [176, 246]. In the present study we demonstrate that maternal dietary folic acid or choline deficiency results in behavioural changes, along with increased apoptosis and alterations in choline metabolism in the cerebellum and hippocampus in *Mthfr*$^{+/+}$ offspring.

6.3 Results

Behavioural Results

Mthfr$^{+/+}$ offspring from FADD or ChDD mothers spent more time ($p<0.05$) with the familiar object during the test trial compared to *Mthfr*$^{+/+}$ offspring from CD mothers (data not shown). The discrimination index, a ratio of time spent with familiar and novel object during the test trial revealed that *Mthfr*$^{+/+}$ offspring from FADD or ChDD mothers spent more time with familiar object during the test trial when compared to *Mthfr*$^{+/+}$ offspring from CD mothers (**Figure 6.1**, $p<0.001$).

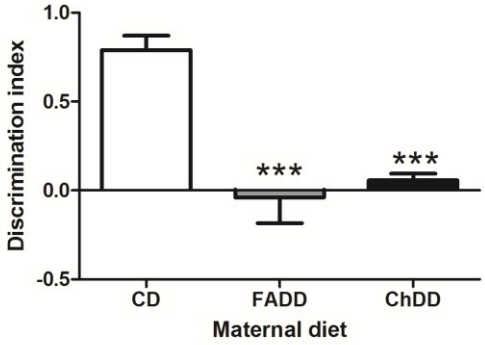

Figure 6.1 The effect of maternal FADD or ChDD diet on novel object recognition task (the discrimination index after one-hour retention period) in 3-week-old *Mthfr*$^{+/+}$ male mice.

Bars represent mean ± standard error of 6 to 9 mice per group. ***p<0.01, unpaired t-test, FADD or ChDD vs. CD.

There was no difference between groups on the footprint pattern task. *Mthfr*$^{+/+}$ offspring from ChDD mothers had a lower movement score when compared to *Mthfr*$^{+/+}$ offspring from CD mothers (**Figure 6.2,** p<0.05)

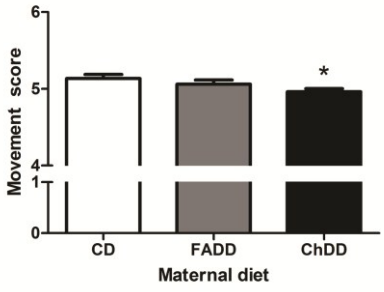

Figure 6.2 The effect of maternal FADD or ChDD diet on movement score of ladder beam walking task (skilled motor) function in 3-week-old *Mthfr*$^{+/+}$ male mice.

Bars represent mean ± standard error of 9 to 12 mice per group. *p<0.05, unpaired t-test, ChDD vs. CD.

Mthfr$^{+/+}$ offspring from FADD mothers crossed more squares in the open field when compared to *Mthfr*$^{+/+}$ offspring from CD mothers (**Figure 6.3A**, $p<0.05$). There was no difference between *Mthfr*$^{+/+}$ offspring from ChDD and CD mothers. *Mthfr*$^{+/+}$ offspring from FADD mothers spent more time in the inside squares of the open field when compared to *Mthfr*$^{+/+}$ offspring from CD mothers (**Figure 6.3B**, $p<0.01$). No difference was observed between *Mthfr*$^{+/+}$ offspring from ChDD and CD mothers.

Prepulse inhibition (PPI) testing revealed no difference in percent inhibition between dietary groups at postnatal day 28 and 60 testing time points.

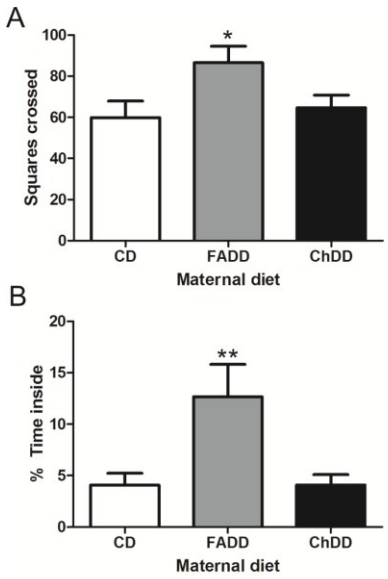

Figure 6.3 The effect of maternal of FADD or ChDD diet on open field in 3-week-old *Mthfr*$^{+/+}$ male mice.

A, number of squares crossed (activity) and B, percent time inside squares (anxiety). Bars represent mean $\pm$ standard of 9 to 12 mice per group. *$p<0.05$, unpaired t-test analysis, FADD vs. CD. **$p<0.01$, unpaired t-test, FADD vs. CD.

Body and brain weight, morphology and plasma homocysteine levels

Mthfr$^{+/+}$ offspring from FADD mothers had a higher body weight when compared to *Mthfr*$^{+/+}$ offspring from CD mothers ($p<0.01$; data not shown), whereas *Mthfr*$^{+/+}$ offspring from ChDD mothers had decreased body weight when compared to *Mthfr*$^{+/+}$ offspring from CD mothers ($p<0.05$; data not shown). No change in brain weight was observed between groups.

Mthfr$^{+/+}$ offspring from FADD mothers showed a trend for elevated plasma homocysteine levels (**Figure 6.4**, $p=0.06$) when compared to the *Mthfr*$^{+/+}$ offspring from CD mothers. There was no difference in plasma homocysteine levels between *Mthfr*$^{+/+}$ offspring from ChDD and CD mothers.

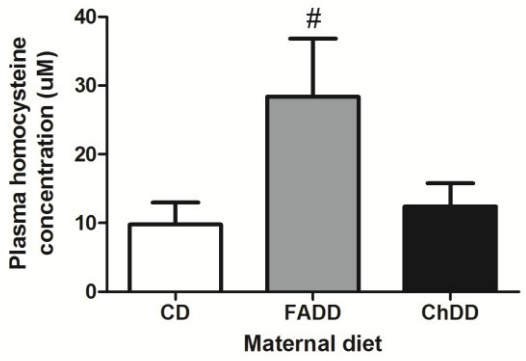

Figure 6.4 Plasma homocysteine concentrations of 3-week-old FADD, ChDD and CD *Mthfr*$^{+/+}$ males.

Bars represent mean ± standard error of 6 mice per group. # $p=0.06$, unpaired t-test, FADD vs. CD.

No difference in cerebellar morphology (foliation and internal granular layer thickness) was observed between groups. *Mthfr*$^{+/+}$ offspring from FADD mothers had no changes in hippocampal morphology, whereas *Mthfr*$^{+/+}$ offspring from ChDD mothers had increased thickness of the CA1 CA3 region (**Figure 6.5A**,

p<0.05) and the dentate gryrus (**Figure 6.5B**, p<0.05) when compared to *Mthfr*$^{+/+}$ offspring from CD mothers.

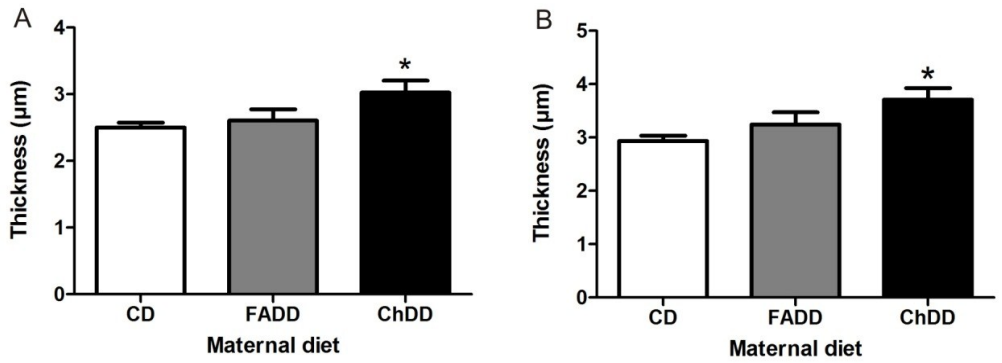

Figure 6.5 The effect of maternal FADD or ChDD diet on hippocampal morphology in 3-week-old *Mthfr*$^{+/+}$ male mice.

A, CA1 CA3 and B, dentate gyrus thickness. Bars represent mean ± standard error of 7-8 mice per groups. *p<0.05, unpaired t-test, ChDD vs. CD.

Cerebellar and hippocampal apoptosis

Increased apoptosis was observed in the cerebellum of *Mthfr*$^{+/+}$ offspring from FADD (**Figure 6.6A,** p<0.05) and ChDD (**Figure 6.6A**, p<0.05) mothers compared to *Mthfr*$^{+/+}$ offspring from CD mothers. In the hippocampus, a trend for increased apoptosis was observed in *Mthfr*$^{+/+}$ offspring from FADD mothers when compared to *Mthfr*$^{+/+}$ offspring from CD mothers (**Figure 6.6B**, p=0.06). Increased apoptosis was observed in *Mthfr*$^{+/+}$ offspring from ChDD mothers compared to *Mthfr*$^{+/+}$ offspring from CD mothers in the hippocampus (**Figure 6.6B,** p<0.05).

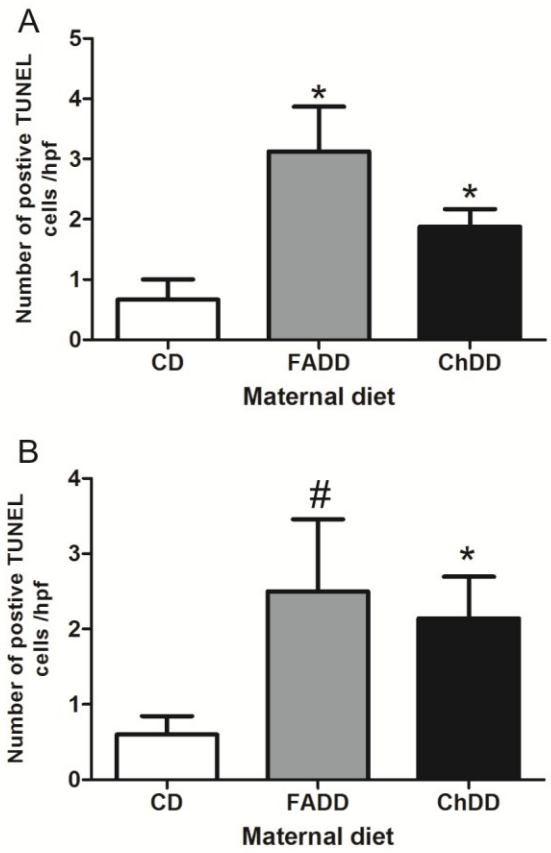

Figure 6.6 Number of apoptotic cells in of 3-week-old *Mthfr*$^{+/+}$ males from mothers fed FADD, ChDD and CD.

A, cerebellum and B, hippocampus. Bars represent mean ± standard error of 6-8 mice per group. *p<0.05, unpaired test, FADD or ChDD vs. CD. # p=0.06, unpaired t-test, FADD vs. CD.

Choline metabolites

In the cerebellum there was decreased amount of betaine in *Mthfr*$^{+/+}$ offspring from ChDD mothers (**Table 6.1**, p<0.05). In the hippocampus *Mthfr*$^{+/+}$ offspring from FADD mothers had decreased levels of betaine (p<0.05) and acetylcholine (p<0.01) when compared to *Mthfr*$^{+/+}$ offspring from CD mothers. *Mthfr*$^{+/+}$ offspring from ChDD mothers had decreased levels of acetylcholine (p<0.05) when compared to *Mthfr*$^{+/+}$ offspring from CD mothers.

Table 6. 1 Concentration of cerebellar and hippocampal choline metabolites in control (CD), folic acid deficient (FADD) and choline deficient (ChDD) diet offspring [1]

	Cerebellum					Hippocampus				
Maternal Diet	CD	FADD	*p* value[2]	ChDD	*p* value[3]	CD	FADD	*p* value[2]	ChDD	*p* value[3]
Metabolite (nmol/g)										
Choline	89 ± 7	102 ± 10	n.s.	100 ± 13	n.s.	60 ± 4.8	79 ± 7	n.s.	72 ± 6	n.s.
Betaine	12 ± 0.5	12 ± 1	n.s.	9 ± 1	p=0.05	6 ± 0.5	5 ± 0.4	p<0.05	5 ± 0.7	n.s.
Acetylcholine	1.7 ± 0.2	1.4 + 0.2	n.s.	1.3 ± 0.2	n.s.	7 ± 0.6	5 ± 0.5	p<0.01	5 ± 0.4	p<0.05

[1] All values are means ± standard error of 6 to 7 mice per group.

[2] *p* values were derived from t-test comparisons between CD and FADD groups.

[3] *p* values were derived from t-test comparison between CD and ChDD groups.

Changes in acetylcholine were followed up with investigating immunoreactive protein levels of choline acetyltransferase (ChAT). Increased protein levels of ChAT were observed in the cerebellum (**Figure 6.7A**) and hippocampus (**Figure 6.7B**) *Mthfr*$^{+/+}$ offspring from both FADD ($p<0.01$; $p<0.05$, respectively) and ChDD ($p<0.01$) mothers when compared to *Mthfr*$^{+/+}$ offspring from CD mothers.

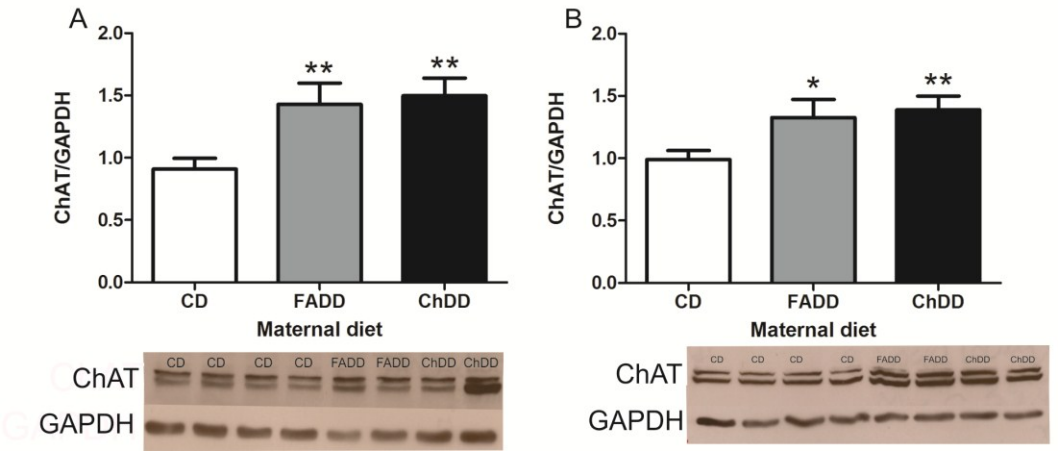

Figure 6. 7. The effect of maternal of FADD or ChDD diet on choline acetyltransferase protein levels in 3-week-old *Mthfr*$^{+/+}$ male mice.

A, cerebellum and B, hippocampus. Bars represent mean ± standard of 6-8 mice per groups.*p<0.05, unpaired t-test, FADD or ChDD vs. CD. **p<0.01, unpaired t-test, FADD or ChDD vs. CD. The panels below the graphs depict a representative Western blot.

6.4 Discussion

In the present study, we found that maternal FADD and ChDD results in changes of brain function in male *Mthfr*$^{+/+}$ offspring. Specifically, *Mthfr*$^{+/+}$ offspring from both ChDD and FADD mothers had impairments in short-term memory. We observed *Mthfr*$^{+/+}$ offspring from ChDD had impaired skilled motor function on the ladder beam walking task, whereas *Mthfr*$^{+/+}$ offspring from FADD mothers were less anxious in the open field. Hippocampal morphological changes were seen in *Mthfr*$^{+/+}$ offspring from ChDD mothers. Increased apoptosis, changes in choline metabolites and increased ChAT protein levels were identified in cerebellum and hippocampus of *Mthfr*$^{+/+}$ offspring from both FADD and ChDD mothers.

Hippocampal function in *Mthfr*$^{+/+}$ offspring from FADD and ChDD mothers

Both folic acid and choline play an important role in hippocampal development *in utero* [115]. The hippocampus is involved in learning and memory [102]. In the present study we observed impaired short-term memory in 3-week-old *Mthfr*$^{+/+}$ offspring from FADD or ChDD mothers. Previous studies in rodents have reported maternal choline deficiency between embryonic days 12 to 17 results in memory impairments as well as changes in hippocampal function [253, 254]. Furthermore, a study in rats reported that *in utero* choline supply affects spatial memory and hippocampal plasticity in adulthood [255]. Maternal choline supplementation during embryonic development has been shown to enhance hippocampal function of offspring [254]. Folic acid deficiencies during pregnancy affect development, specifically a study by [256] reported that folic acid deficiency during pregnancy effects maze learning in second generation rats. In the current study, we observed increased thickness of the CA1 CA3 and dentate gyrus within the hippocampus in *Mthfr*$^{+/+}$ offspring from ChDD mothers. This result may be compensating for increased apoptosis also observed in these animals. We suggest that the cells in the hippocampus of *Mthfr*$^{+/+}$ from ChDD mothers may be compensating for increased demand during choline deficiency.

Increased apoptosis in cerebellum of *Mthfr*$^{+/+}$ offspring

Apoptosis in the brain is required during early postnatal development to remove excess cells in the brain, however too much apoptosis may not be ideal for brain development [200]. Maternal FADD during late gestation (embryonic days 12 to 17) results in increased apoptosis and negatively affects proliferation in fetal

brain tissue [169]. Folate concentrations in maternal plasma were lower in FADD mothers as well as in fetal brain tissue from FADD mothers [169]. The same research group reported similar changes in apoptosis and proliferation in offspring from ChDD mothers [257, 258]. In the present study we observed increased apoptosis in the cerebellum and hippocampus of *Mthfr*$^{+/+}$ offspring from both FADD and ChDD mothers. Interestingly increased levels of apoptosis have been associated with elevated levels of plasma homocysteine [138, 139]. In the current study we observed increased plasma homocysteine in *Mthfr*$^{+/+}$ offspring from FADD mothers, no change was observed in offspring from ChDD mothers. Additionally, our group has previously shown that pregnant females maintained on FADD (32.8±9.2 μM) or ChDD (40.1±2.9 μM) do indeed have elevated levels of plasma homocysteine when compared to CD (13.8±2.2 μM) mothers [164, 172, 176, 246]. Increased apoptosis observed in the present study may be a result of elevated maternal or offspring plasma homocysteine levels.

In the present study we observed increased apoptosis in cerebellum and hippocampus of *Mthfr*$^{+/+}$ offspring from FADD or ChDD mothers. Elevated levels of homocysteine have been shown to result in more oxidative stress [259]. These increased levels may contribute to the increased apoptosis through elevated levels of DNA damage or excitatory mechanisms, such as repeated stimulation of the NMDA receptor [136] or increased levels of intracellular calcium [229]. Elevated levels of homocysteine can also be a result of nutrientional deficiencies (e.g. folate) and since folate plays an important role in DNA repair and proliferation, this may also lead to cell death.

Behavioural changes in *Mthfr*$^{+/+}$ offspring from FADD and ChDD mothers

A previous study has shown that adult CD-1 offspring from FADD mothers had changes in anxiety, specifically these mice showed increased anxiety behaviour on the elevated plus maze [168]. In the present study we observed decreased anxiety in *Mthfr*$^{+/+}$ offspring from FADD mothers on the open field task. The discrepancy between the two studies may be due to the fact that the study by [168] tested adult mice, whereas in the present study we tested 3-week-old mice. Furthermore, the authors of [168] removed pregnant females from FADD on gestational day 18, whereas in our study we maintained mothers on diets for the duration of pregnancy and lactation. Lastly, both studies differed in the amount of folic acid in diets.

In *Mthfr*$^{+/+}$ offspring from ChDD mothers, we observed impaired motor function. Alterations in motor function have not been reported as a result of choline deficiency, whereas choline supplementation has been previously shown to improve motor function in Rett syndrome mutant mice [260]. This may be due to increased expression of a neurotrophin, nerve growth factor, in the striatum of mice [112].

Epidemiological studies have reported that elevated levels of plasma homocysteine are associated with schizophrenia [143, 261-263]. In the present study we investigated this association using a mouse model. A common trait in schizophrenia is lack of inhibition[264]. A PPI assay can test inhibition in humans and mice [264]. In the present study we did not observe a difference in percent inhibition on the PPI task between groups when we tested mice at 28 days and 60 days post-natal.

Choline metabolites in cerebellum and hippocampus

Folate and choline metabolism are both linked by remethylation of homocysteine to methionine [38]. In the brain choline is required for a number of processes, including lipid metabolism and synthesis of Ach [63]. Ach is involved in both cognitive and motor function [106]. In the present study we observed changes in choline metabolites in the cerebellum of *Mthfr*$^{+/+}$ offspring from ChDD mothers and in the hippocampus of *Mthfr*$^{+/+}$ mice from both FADD and ChDD mothers. Decreased levels of Ach and increased levels of ChAT were observed in the hippocampus of *Mthfr*$^{+/+}$ offspring from FADD or ChDD mothers. Increased levels of ChAT have previously been shown to compensate for decreased levels of acetylcholine [207]. Prenatal availability of choline has been reported to affect the hippocampal cholinergic system, specifically maternal choline deficient diet increased levels of ChAT and acetylcholinesterase (AChe) in the hippocampus possibly to increase synthesis of Ach [250]. Another study described increase choline transporter expression in the hippocampus, including the dentate gyrus of young and adult rats as a potential mechanism to increase choline availability as a result of prenatal choline deficiency [265]. Interestingly, in rats maternal dietary choline supplementation from embryonic day 11 to 17 has been shown to reverse the negative effects of maternal folic acid deficiency on neurogenesis and apoptosis [111].

In summary we observed behavioural changes in *Mthfr*$^{+/+}$ offspring from FADD and ChDD mothers. These changes in behaviour may be a result of increased maternal or offspring plasma homocysteine, apoptosis or altered choline metabolism in the cerebellum and hippocampus of 3-week-old *Mthfr*$^{+/+}$ offspring.

6.5 Acknowledgements

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Chapter VII: General Discussion

7.1 Role of folate metabolism in CNS

Epidemiological studies have shown that deficiencies in folate metabolism, either genetic [143, 266] or dietary [68] are associated with neurological disturbances. This thesis used a mouse model to evaluate the effects of genetic (e.g. MTHFR and MTRR) on brain function and structure in adult mice, as well as the effect of maternal genetic and dietary deficiencies in folate metabolism on offspring brain function and structure.. The hippocampus, primarily responsible for learning and memory [102], was shown to be significantly affected by genetic as well as maternal genetic and dietary deficiencies in the folate metabolism when compared to the cerebellum. Specifically, in both MTHFR and MTRR-deficient male mice there are changes in hippocampal function (e.g. impaired short-term memory), reduced volume and global DNA hypomethylation as well as altered choline metabolites in the hippocampus. In the maternal studies we observe impaired short-term memory along with increased apoptosis in hippocampus and changes in choline metabolites. There is significant amount of neurogenesis during prenatal and early postnatal brain development [199]. Neurogenesis in the adult brain is minimal, however, the hippocampus [104] and cerebellum [105] are exceptions. Since folates play an important role in the generation of new cells, a disruption in folate metabolism may alter homeostasis in these two structures, leading to dysfunction.

Folate metabolism provides one carbon groups to facilitate the generation of *S*-adenosylmethionine (SAM), a global methyl donor for many reactions in the body [30]. In the brain SAM plays a role in the generation of neurotransmitters as well as lipid metabolism [30, 114]. Additionally, epigenetic modifications (e.g.

DNA methylation) have been reported to regulate neurogenesis in the hippocampus [267]. Maintenance of DNA methylation is needed to conserve neuronal identity, for example methyl CpG binding protein 2 (MeCP2) is expressed in mature neurons and is involved in suppressing expression of glial-specific genes in neurons [268]. Furthermore, when MeCP2 is over-expressed then there is an increase in neuronal differentiation in neural stem cells [269]. Global DNA hypomethylation in hippocampal and cerebellar tissue in *Mthfr*$^{-/-}$ and *Mtrr*$^{gt/gt}$ mice was described in this thesis. A disruption in methylation may interfere with homeostasis in the brain possibly leading to changes in function.

Genetically modified mouse models have been developed to study *in vivo* the effects of elevated levels of plasma homocysteine. These models include genetic deficiencies in MTHFR, MTRR, betaine homocysteine methyltransferase (BHMT), and cystathionine-β-synthase (CBS) [270]. The concentration of plasma homocysteine in *Mthfr* knockout mice is ~60 μM [222] and *Mtrr*-deficient mice is ~12 μM [34]. *BHMT* knockout mice have plasma homocysteine concentrations of ~50μM [271], these mice were recently developed therefore the brain function and structure has not been investigated. Transsulfuration is an alternative pathway to reduce levels of homocysteine [39], in this reaction CBS converts homocysteine to cystathionine. A study by [39] described the CBS knockout mouse model. *CBS*$^{-/-}$ mice have significantly elevated plasma homocysteine levels (~200 μM) and a low survival rate. The mice that do survive, do not develop neuropathologies [39, 270], this may be because *CBS*$^{-/-}$ mice do not have DNA methylation inhibition in brain tissue [39, 272], as reported in MTHFR [35, 96, 222] and MTRR (Chapter IV) mouse models.

7.2 Role of elevated homocysteine on brain function and neurodegeneration

Homocysteine has been reported to damage cells of the CNS both *in vitro* and *in vivo* [136, 138, 150, 222]. Three possible mechanisms of action have been proposed, the first is that homocysteine leads to neurotoxicity. Secondly, increased levels of homocysteine disrupt one carbon metabolism and results in accumulation in *S*-adenosylhomocysteine (SAH), inhibiting methylation reactions as discussed in previous section. Lastly homocysteine can damage the CNS through vascular damage [270].

In vitro data has reported that homocysteine leads to apoptosis [136] and in this thesis we present *in vivo* data that elevated plasma levels result in increased apoptosis within the hippocampus and cerebellum during early postnatal development. A possible mechanism through which homocysteine may induce cell death in the brain is via the NMDA receptor. Glutamate is an excitatory neurotransmitter in the brain and homocysteine is thought to act as an agonist for a glutamate binding subunit on the NMDA receptor causing repeated excitatory stimulation [138, 139, 273]. Indeed, homocysteine and its' derivatives have been reported to be glutamate receptor agonists [274]. Another possibility of homocysteine neurotoxic effects is through increasing intracellular calcium levels [275]. The Rozen laboratory has reported that the expression of genes involved in intracellular calcium signalling in brain tissue are altered in young BALB/c *Mthfr*$^{-/-}$ mice [151]. Lastly, increased oxidative stress and reactive oxygen species may be a result of hyperhomocysteinemia [136, 276]. Oxidative damage has been reported in the plasma of patients with homocystinuria [277], possibly resulting in vascular damage [276].

Lastly, epidemiological evidence indicates that elevated levels of plasma homocysteine play a role in the development of dementia and other neurodegenerative disorders [137, 218, 278]. This is not surprising given that elevated levels of homocysteine are common in the elderly population [233]. Epidemiological studies have reported that elevated levels of plasma homocysteine are associated with brain atrophy, specifically in the hippocampus, as well as total brain atrophy [203, 227, 279]. We observed total brain atrophy in severe-MTHFR deficient animals [222], as well as atrophy in the hippocampus of *Mthfr*$^{-/-}$ [222] and *Mtrr*$^{gt/gt}$ mice. Controls or individuals with MCI that undergo homocysteine lowering treatment (e.g. dietary B-vitamin supplements) show decreased rate of brain atrophy as well as slowed cognitive decline [124, 225, 232]. This thesis identified that 3-month-old MTRR deficient mice have impairments in short-term memory. Reduced hippocampal volume and decreased levels of Ach have been reported to play a role in the process of neurodegeneration [233, 280], akin to what we observed in *Mtrr*$^{gt/gt}$ mice in chapter IV of this thesis. Furthermore, *Mtrr*$^{gt/gt}$ mice have mildly elevated plasma homocysteine levels, making them an attractive animal model to study the mechanism of how the folate metabolism may be involved in neurodegeneration and potentially, the development of therapies.

7.3 Importance of choline for normal brain function

In 2000, choline was designated an essential nutrient [160]. The importance of choline was reiterated in this thesis, especially for brain function. Genetic deficiencies in MTHFR or MTRR as well as maternal deficiencies in folic acid and choline in *Mthfr*$^{+/+}$ offspring revealed altered choline metabolism in the cerebellum

and hippocampus. In the brain choline plays a role in the synthesis of the neurotransmitter, acetylcholine and as well as membrane synthesis. Choline and its' metabolite betaine cannot reduce plasma homocysteine levels in brain because BHMT is not expressed in the brain [38]. In this thesis we consistently observed decreased levels of Ach. We propose that in the cerebellum and hippocampus of deficient mice there is a decrease of oxidation of choline to betaine, so that choline can be made available for acetylcholine synthesis as well as membrane synthesis [222]. Increased protein levels of ChAT, an enzyme involve in Ach synthesis, were also observed in cerebellum and hippocampus of deficient mice. During Ach deficiency levels of ChAT have been reported to increase [207]. When a patient with severe MTHFR deficiency was treated with betaine, it resulted in normal levels of homocysteine and methionine [80]. Additionally, when pregnant *Mthfr*$^{+/-}$ female mice were fed betaine in drinking water some ameliorations in neurological phenotype of *Mthfr*$^{-/-}$ offspring was reported [152]. Data from this thesis suggests that choline may also be beneficial in the treatment of neurological symptoms associated with MTHFR, MTRR or maternal folic acid or choline deficiency.

7.4 Maternal contributions of folic acid and choline during pregnancy

It is well known that supplementation for folic acid is necessary during pregnancy for closure of the neural tube [68]. Mouse studies have shown that choline is essential for the closure of the neural tube [115]. However, the long-term effects of these deficiencies during early fetal and neonate development on brain function of offspring remain unclear. Few epidemiological studies have reported negative behavioural outcomes in children from folic acid deficient mothers [166, 243, 281]. One study shows no effect of folic acid deficiency on behaviour of

offspring possibly due to low socio-economic status of the population studied [241]. In this thesis, using a mouse model, we reported changes in behavioural function, along with increased apoptosis and changes in choline metabolites in cerebellum and hippocampus of *Mthfr*$^{+/+}$ offspring from mothers deficient in MTHFR or mothers with dietary deficiencies in folic acid or choline. Interestingly, another study reported that maternal deficiencies during pregnancy result in changes in affective behaviours and memory function of offspring during adulthood [243]. Recent data has shown the negative impact of deficiencies in early fetal and neonatal development [159]. For example the 'fetal origins hypothesis' which suggests that disorders later in life may originate from malnutrition during early fetal, neonatal and childhood development [282]. There is a significant amount of plasticity during development that any disruption may lead to changes in programming of tissues and organs [156, 282]. One study reported that in pregnant female rats maintained on a low methyl diet there was an accumulation of homocysteine in both the cerebellum and hippocampus of offspring [243]. Adequate levels of folates and choline are required during pregnancy not only for closure of neural tube of offspring, but also for normal brain function as reported in this thesis.

7.5 Future directions

In chapter III we identified that a severe MTHFR deficiency resulted in significant changes in hippocampal function. In addition we identified changes in choline metabolites in hippocampal and cerebellar tissue. Dietary supplementation with choline during fetal development or adulthood may result in ameliorations of neurological symptoms in mice. It may be worthwhile to investigate choline

metabolites in other tissues (e.g. liver) and plasma of *Mthfr*$^{-/-}$ mice to determine if there is redistribution of choline as we described in MTRR-deficient mice. We did not observe any behavioural changes in *Mthfr*$^{+/-}$ mice. This may be a result of strain differences, since the C57BL/6 mice have a less severe phenotype when compared to the BALB/c mice [96, 188]. It may be interesting if brain function and structure was investigated in BALB/c heterozygotes and wild-type mice. Changes in motor function and affective behaviours have already been described [154, 283] in these mice, however, the effect of MTHFR deficiency on hippocampal function has not yet been investigated.

In chapter IV we identified that 3-month-old MTRR-deficient mice have impairments in short-term memory, specifically in the novel object recognition task and y-maze. Testing long-term memory, using the standard Morris water maze will provide additional information on the severity of memory impairment in these mice. Additionally, the radial arm maze could also be used to test spatial memory. Supplementing MTRR-deficient mice diets with choline may provide to be beneficial because of the changes in choline metabolites observed in brain tissue of these mice. Increased plasma homocysteine levels have been associated to neurodegeneration in humans [233]. *Mtrr*$^{gt/gt}$ mice have elevated levels have mildly elevated levels of homocysteine (~12μM), therefore testing brain function and biochemistry of aged (e.g. 6 months or one year) mice will help to understand how a genetic deficiency in folate metabolism and mildly elevated levels of plasma homocysteine affect the course of neurodegeneration. *Mthfr*$^{+/-}$ mice have approximately the same plasma homocysteine concentration (~14μM). In chapter three we observed no significant changes in brain function and structure of

C57BL/6 *Mthfr*$^{+/-}$ mice. As mentioned earlier, other studies using *Mthfr*$^{+/-}$ mice on the BALB/c background have reported changes in brain function [283]. Therefore investigating brain function in aged BALB/c *Mthfr*$^{+/-}$ mice may also assist in the understanding of how a genetic deficiency in folate metabolism and elevated plasma homocysteine affect the course and mechanisms involved in neurodegeneration.

In chapter V we identified some preliminary data that suggests a maternal MTHFR deficiency results in altered gait, motor function and short-term memory. In this study, mothers were maintained on a CD which contains 2mg/kg of folic acid, whereas standard laboratory mouse chow contain approximately 6-9mg/kg of folic acid (Harlan Teklad, Indianapolis, IN). The amount of folic acid in the CD is in the lower range for mouse diet and might have contributed to brain function changes observed in offspring from *Mthfr*$^{+/-}$ mother. These preliminary data is interesting since the *Mthfr*$^{+/-}$ mice mimic the polymorphism at base pair 677C→T, which is present in 5-20% of the Northern American and European populations [87], however, additional behavioural testing should be done to confirm these findings. For example, animals could be tested on the Morris water maze to determine whether there are also impairments in long-term memory. Animals could also be tested on another motor task (e.g. catwalk or rotarod) to confirm motor impairments reported in this thesis. Increased brain apoptosis was observed in 3-week-old *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mothers, it may be interesting to evaluate neurogenesis to determine if more cells were being generated as a result of increased cell death during early postnatal development.

In chapter VI we observed short-term memory impairments in *Mthfr*$^{+/+}$ offspring from FADD and ChDD mothers. Another group of mice could be tested on another short-term memory task (e.g. y-maze) to confirm the findings. Furthermore, other memory tests (e.g. long-term memory) could be used in these mice. *Mthfr*$^{+/+}$ offspring from ChDD mothers had impaired motor function; this finding could be confirmed by testing another group on the same task or another motor task (e.g. rotarod task or catwalk). Increased thickness in the cellular layer of the CA1 CA3 and DG regions was observed in *Mthfr*$^{+/+}$ offspring from ChDD mother, this is an interesting finding since we also identified increase apoptosis in the hippocampus of the same mice. Investigating neurogenesis in these mice may help understand why there are more cells in these areas. Recent work from our group has shown that pregnant females on FADD and ChDD diets have altered inflammation in placenta, liver and spleen tissue [176, 246]; it may be interesting to investigate inflammation in offspring tissue. Lastly, it may be worthwhile to analyze brain function and structure of *Mthfr*$^{+/-}$ offspring from FADD or ChDD mothers. I hypothesize that the phenotype would be more severe in *Mthfr*$^{+/-}$ offspring, since previous work has reported increased plasma homocysteine in heterozygote mice [35] and there would be a 'double hit' impairing homocysteine metabolism.

In conclusion this thesis identified that a genetic deficiency in MTHFR or MTRR results in changes in brain function. As well, maternal contributions of MTHFR, folic acid or choline play an important role in the development of brain function in wild-type offspring. Overall, changes in brain function may be a result

of altered choline metabolism, increased apoptosis or global DNA hypomethylation.

7.6 Claims to originality

1. Three-month-old C57BL/6 *Mthfr*$^{-/-}$ males have impaired short-term memory in the novel object recognition and y-mazes tasks as well as long-term memory in the novel object recognition task. Altered gait in footprint pattern task and impaired motor function in ladder beam walking task. Increased exploratory behaviour in open field task and decreased anxiety in open field and elevated plus maze task.

2. Reduced total brain volume in *Mthfr*$^{-/-}$ mice. Smaller hippocampal and cerebellar volume when corrected for brain volume in *Mthfr*$^{-/-}$ mice.

3. *Mthfr*$^{-/-}$ thinner CA1 CA3 and dentate gyrus cell layers in the hippocampus

4. Increased choline acetyltransferase protein (ChAT) protein levels in cerebellum and hippocampus of *Mthfr*$^{-/-}$ mice

5. Increased mRNA and protein levels of glucocorticoid receptor (GR) in hippocampus of *Mthfr*$^{-/-}$ mice.

6. Three-month-old male *Mtrr*$^{gt/gt}$ mice have impaired short-term memory in novel object recognition and y-maze tasks, altered gait in footprint pattern task and increased exploration and decreased anxiety in open field task.

7. *Mtrr*$^{gt/gt}$ mice have increased ChAT protein levels in cerebellum and hippocampus.

8. *Mtrr* $^{gt/gt}$ mice have decreased mRNA expression of *CHDH* and *BHMT* in liver.

9. Three-week-old male *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mothers have impairments in short-term memory in novel object recognition, altered gait on footprint pattern and motor function on ladder beam walking tasks.

10. Increased apoptosis in hippocampus and cerebellum of *Mthfr*$^{+/+}$ offspring from *Mthfr*$^{+/-}$ mothers.

11. Three-week-old *Mthfr*$^{+/+}$ offspring from folic acid (FADD) and choline (ChDD) deficient diet mothers showed impairments in short-term memory on novel object recognition task.

12. *Mthfr*$^{+/+}$ offspring from ChDD mothers have impairments in skilled motor function on ladder beam walking task.

13. *Mthfr*$^{+/+}$ offspring from FADD mothers were more active and have decreased anxiety on open field task.

14. *Mthfr*$^{+/+}$ offspring from ChDD mothers have thicker cell layers in CA1 CA2 and DG in the hippocampus.

15. *Mthfr*$^{+/+}$ offspring from FADD and ChDD mothers have increased apoptosis in cerebellum and hippocampus.

16. *Mthfr*$^{+/+}$ offspring from FADD and ChDD mothers have increased ChAT protein levels in cerebellum and hippocampus.

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